

**Pincher-generated Nogo-A signalosomes mediate growth cone collapse
and retrograde transport**

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Summary

The ability of adult CNS neurons to successfully regenerate their axons upon injuries such as stroke and spinal cord injury is very limited. Over the last two decades researches have focused on the challenging question of how to overcome this regenerative failure. The identification of the myelin associated neurite outgrowth inhibitor Nogo-A shed light on the inhibitory nature, compared to the PNS, of CNS myelin. Subsequent Nogo-A neutralization experiments led to successful CNS regeneration in animal models of spinal cord injury. The first part of Chapter 1 summarizes important findings regarding Nogo-A function as a neurite outgrowth inhibitor and gives insight into emerging roles of Nogo-A during development and disease.

Nogo-A contains two inhibitory regions for neurite outgrowth inhibition: the Nogo66 region, also common to Nogo-B and Nogo-C, and the Nogo-A specific region, Nogo 20. Whereas the signaling mechanisms of the Nogo66 region are well characterized, comparatively little is known about the signaling complex of Nogo 20. Since blocking of the Nogo66 receptor complex does not completely abolish myelin inhibition of neurite outgrowth, it is of great importance to elucidate the Nogo66 receptor independent mechanism of Nogo-A signaling.

Over the last few years it has become clear that endocytosis plays an active role during cellular communication. A number of surface ligand-receptor complexes are known to internalize into cells and signal from endosomes. The second part of Chapter 1 introduces endocytosis as a possible mechanism for Nogo 20 signaling and reviews the known endocytic pathways.

In Chapter 2 we find that the soluble, active Nogo 20 fragment is internalized into neuronal cells. The internalization into early endosomes does not follow a conventional clathrin-and dynamin dependent route, it rather depends on the Pincher protein and the small GTPase Rac. This Pincher-mediated macroendocytosis of Nogo 20 results in the formation of Nogo 20 signalosomes that direct RhoA activation and growth cone collapse. In compartmentalized chamber cultures, Nogo 20 is endocytosed into neurites and retrogradely transported to the cell bodies of DRG neurons, triggering RhoA activation en route and decreasing pCREB levels in

cell bodies. The decrease of pCREB points towards an antagonistic regulation of the growth machinery by Nogo-A and neurotrophins. Taken together, the findings of the present thesis suggest that Pincher-dependent macroendocytosis may lead to the formation of a Nogo-A signaling endosome that is retrogradely transported from the axons to the cell body thus suppressing the neuronal growth program.

Zusammenfassung

Die Nervenzellen des adulten Zentralnervensystems können ihre verletzten Axone nach Hirn- und Rückenmarksverletzungen nur beschränkt regenerieren. Der Grund für dieses limitierte Wachstumspotential blieb der Wissenschaft lange Zeit ein Rätsel. Die Identifizierung des wachstumshemmenden Faktors Nogo-A, welcher mit der Myelinschicht assoziiert ist, gab Aufschluss über die hemmende Natur des Zentralnervensystems im Vergleich zu dem zur Regeneration befähigten peripheren Nervensystems. In verschiedenen Tiermodellen konnte aufgezeigt werden, dass eine Neutralisierung von Nogo-A zur erfolgreichen Regeneration des Zentralnervensystems führt. Der erste Teil von Kapitel 1 gibt eine Übersicht über die Funktion von Nogo-A als Wachstumshemmer, sowie neue Rollen von Nogo-A während der Entwicklung und in der Pathologie.

Zwei Regionen des Nogo-A Moleküls tragen zur wachstumshemmenden Wirkung bei: die Nogo66 Region, welche auch in Nogo-B und Nogo-C vorkommt, sowie die Nogo-A spezifische Region, Nogo 20. Der Signalkomplex von Nogo66 ist gut charakterisiert, über Nogo 20 hingegen ist noch wenig bekannt. Weil die Blockade des Nogo66 Rezeptorkomplexes die wachstumshemmende Wirkung von Myelin nur teilweise aufheben kann, ist es von enormer Wichtigkeit, den Nogo 20 Mechanismus zu ergründen. Eine Reihe von Studien belegt, dass die Endozytose eine wichtige Rolle für die zelluläre Kommunikation spielt.

Einige Ligand-Rezeptor Komplexe werden von der Zelloberfläche internalisiert und übermitteln auf diese Weise die Signale von Aussen ins Zellinnere. Der zweite Teils von Kapitel 1 führt die Endozytose als möglichen Mechanismus für die Nogo 20 Signalübermittlung ein and erläutert die verschiedenen endozytotischen Wege.

In Kapitel 2 zeigen wir, dass das aktive Nogo-A Fragment, Nogo 20, in die neuronalen Zellen internalisiert wird. Die Internalisierung in die frühen Endosomen folgt nicht über den konventionellen Clathrin- und Dynamin-abhängigen Weg, sondern über das Pincher Protein und die kleine GTPase Rac. Diese Pincher Protein-abhängige Makroendozytose von Nogo 20 führt

zur Bildung von Nogo 20 Signalosomen, welche dann die Aktivierung von RhoA und den Kollaps des Wachstumskegels steuern. Zudem zeigen wir, dass das Nogo 20 von den Nervenfortsätzen aufgenommen und zum Zellkörper zurücktransportiert wird. Auf dem Weg zum Zellkörper aktiviert es die GTPase RhoA und reduziert die Menge an phosphoryliertem CREB. Diese Ergebnisse bezüglich pCREB weisen auf einen antagonistischen Mechanismus zwischen Nogo-A und den Neurotrophen hin.

Zusammengefasst deuten die Befunde der vorliegenden Arbeit darauf hin, dass das mit Myelinschicht assoziierte Nogo-A Protein zur Bildung von Signalosomen führt, welche zum Zellkörper zurücktransportiert werden, um das neuronale Wachstumsprogramm zu unterdrücken.

Chapter 1

“The art and science of asking questions is the source of all knowledge.”

Thomas Berger

Overview

The introduction of the present thesis gives an overview of two topics. The first part reviews the current knowledge about the myelin-associated neurite growth inhibitor Nogo-A. The second part summarizes the known endocytic pathways and the possible role of endocytosis for Nogo-A signaling.

1.1 Nogo-A as a myelin-associated neurite growth inhibitor in the CNS

The ability of the adult mammalian central nervous system (CNS) to undergo functional and anatomical recovery following large injuries is very limited. Although spontaneous plastic rearrangements of neuronal circuits can occur, the functional recovery of CNS compared to the PNS is very poor (Bareyre et al., 2004; Edgerton et al., 2004; Payne and Lomber, 2001; Raineteau and Schwab, 2001; Sanes and Donoghue, 2000). This difference in repair between CNS and PNS was for a long time attributed to the absence of growth factors in CNS. However, the discovery of brain-derived neurotrophic factor (BDNF) (Barde et al., 1982; Leibrock et al., 1989), neurotrophin NT-3 (Hohn et al., 1990; Rosenthal et al., 1990) and NT-4/5 (Berkemeier et al., 1991) in adult CNS changed this view. The demonstration that CNS myelin in contrast to PNS myelin is inhibitory for axonal growth and the characterization of the inhibitory CNS white matter components NI 35 and NI 250 (Caroni and Schwab, 1988a; Caroni and Schwab, 1988b) led to a new picture, in which the presence of growth inhibitory molecules specifically localized in CNS white matter prevent successful regeneration (Schwab and Thoenen, 1985). This view was further supported by studies showing functional recovery and axonal regeneration after spinal cord injury by the neutralization of NI 250 with the IN-1 antibody (Bregman et al., 1995; Schnell and Schwab, 1990; Thallmair et al., 1998). Shortly after, NI 250 has been identified and renamed Nogo-A, a 1163 amino acids large transmembrane protein (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000; Spillmann et al., 1998). In the last decade, numerous studies have shown that the suppression of Nogo-A signaling by either Nogo-A neutralization or blocking of the Nogo receptor (NgR) components or inhibition of the known downstream signaling machinery such as Rho-A and Rho-A kinase (ROCK) leads to enhanced regeneration

and compensatory nerve fibre growth resulting in increased functional recovery of adult mammalian CNS after traumatic or stroke injuries (Cafferty and Strittmatter, 2006; Schwab, 2004; Yiu and He, 2003). Over the past years, several other growth inhibitory molecules have been identified: some of them in myelin such as MAG (myelin-associated glycoprotein), OMgP (oligodendrocyte myelin glycoprotein), and members of the ephrin, semaphorin and netrin families, others in the astroglial scar such as CSPGs (chondroitin sulphate proteoglycans) (Carulli et al., 2005; Low et al., 2008; Maier and Schwab, 2006; Pasterkamp and Verhaagen, 2006; Schwab, 2004; Yiu and He, 2006). However, Nogo-A has attracted the most interest. In the following sub-chapters I highlight functional roles of Nogo-A, its family and the signaling components.

The Nogo/Reticulon family

Nogo-A belongs to the reticulon (RTN) protein family and is also known as RTN-4A. RTNs are a class of integral membrane proteins enriched in the endoplasmatic reticulum (ER). They are ubiquitously expressed in all eukaryotes and characterized by a RHD (reticulon homology domain) consisting of two transmembrane domains flanking a hydrophilic loop. In mammalian cells, four RTN genes are present and several splice variants for each of them have been identified. In humans, the *nogo/rtn 4* gene encodes three distinct proteins (Nogo-A, Nogo-B and Nogo-C), which arise by alternative splicing (Nogo-A, -B) or alternative promoter usage (Nogo-C) (Figure 1) (Oertle and Schwab, 2003).

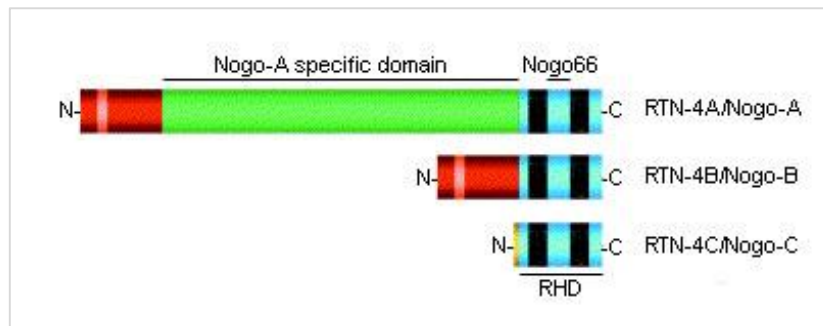


Figure 1: Schematic representation of the mammalian *nogo* gene adapted from Oertle and Schwab, 2003

In recent years RTNs have been linked to the formation and maintenance of the highly curved tubular ER (De Craene et al., 2006; Hu et al., 2008; Shibata et al., 2008). Their over-expression generates long, unbranched and bundled tubules, and their deletion leads to disruption of peripheral tubular ER (Voeltz et al., 2006). In addition, it has been demonstrated that lack of RTNs blocks nuclear pore complex (NPC) formation suggesting a role for RTNs in NPC assembly (Dawson et al., 2009). A number of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins i.e. syntaxin 1, syntaxin 7, syntaxin 13 and VAMP2 but also Sec6p (Voeltz et al., 2006) were found to interact with RTNs suggesting their role in vesicle trafficking events, including exocytosis (Steiner et al., 2004). The over-expression of RTN3 has been directly implicated in ER-Golgi transport (Wakana et al., 2005). In response to ER stress RTN were also linked to apoptosis (Kuang et al., 2005; Teng and Tang, 2008).

Very little is known about the membrane topology and the native tertiary structure of Nogo-A. A few studies suggest that Nogo-A exhibits an unusual membrane topology having the N-terminal part and the Nogo66 loop exposed to the cell surface (GrandPre et al., 2000; Oertle et al., 2003; Voeltz et al., 2006). Circular dichroism (CD) and nuclear magnetic resonance (NMR) studies have addressed the tertiary structure of Nogo-A. So far, only parts of the Nogo-A protein have been characterized. The Nogo66 fragment adopts a helical conformation consisting of three alpha helices (Li et al., 2008; Li et al., 2006; Zander et al., 2007) The N-terminal part, however, is mostly unstructured and exhibits a small content of beta sheet (Zander et al., 2007). The presence of structural disulfide bridges supports an extracellular location of the N terminal Nogo-A fragment (Zander et al., 2007).

The role of Nogo-A beyond the inhibition of CNS nerve fiber regeneration

Besides its role in the injured CNS, Nogo-A has been also described as a general suppressor of growth and plasticity in the intact CNS. Nogo-A neutralization, in adult rats using monoclonal antibodies, induces a transitory growth response (Bareyre et al., 2002; Buffo et al., 2000; Zagrebelsky et al., 1998). In addition, genetic ablation of Nogo-A results in an enhanced expression of the neuronal growth related proteins, which in turn leads to enhanced growth cone motility by regulation of the actin cytoskeleton through modulation of the LIM kinase/cofilin pathway (Montani et al., 2009). Further, it has been shown that the plasticity of the visual cortex ocular dominance columns is extended beyond the normal post-natal critical period in mice lacking the Nogo66 receptor NgR or Nogo-A/B (McGee et al., 2005).

Another role of Nogo-A, although less explored, is found during CNS and PNS development. Mignorance-Le Meur and co-workers have suggested that Nogo-A may play a role during corticogenesis by modulating tangential neuronal migration, and neurite formation and maturation (Mignorance-Le Meur et al., 2007). Similarly, zebrafish embryos lacking orthologues of Nogo and Nogo receptor show severe defects in axon path finding during PNS development. Their peripheral nerves are significantly shorter, defasciculated, and project into inappropriate target areas (Brosamle and Halpern, 2009).

Nogo-A is also implicated in several neurological diseases. For instance, increased Nogo-A and Nogo-B levels have been found in the skeletal muscles of patients with amyotrophic lateral sclerosis (ALS), a motor neuron disease leading to progressive skeletal muscle atrophy (Dupuis et al., 2002; Jokic et al., 2005). The genetic ablation of Nogo-A reduces muscle denervation and prolongs survival in a mouse model of ALS whereas the over-expression of Nogo-A promotes denervation and destabilizes motor nerve terminals (Jokic et al., 2006). In addition, soluble Nogo-A fragments have been detected in cerebrospinal fluid (CSF) of patients with multiple sclerosis (MS), an autoimmune inflammatory disease characterized by the demyelination of CNS axons. Neutralization of Nogo-A by either an active or passive vaccination as well as genetic ablation resulted in reduced axonal damage and fewer overall inflammatory lesions in EAE, a

mouse model of MS (Karnezis et al., 2004). Accumulation of Nogo-A has further been found in β -amyloid plaques of Alzheimer's disease (AD) brains (Gil et al., 2006). The association of Nogo-A with BACE, β -amyloid precursor protein cleavage enzyme, supports the possible role of Nogo-A in AD (Wojcik et al., 2007). However, it is unclear whether in AD Nogo-A plays a more protective or a degenerative role. Another disease, in which Nogo-A may be involved is schizophrenia. In the cortex of schizophrenic patients, Nogo-A levels have been shown to be elevated (Novak et al., 2002).

Nogo-A downstream signaling

Nogo-A is predominantly found in the innermost, adaxonal as well as in the outermost myelin membrane in oligodendrocytes (Huber et al., 2002; Wang et al., 2002b). The neuronal expression is highest during development but is maintained in particular neuronal populations in adults (Huber et al., 2002; Wang et al., 2002a).

Nogo-A is a large membrane protein of 1163 amino acids with two main inhibitory regions for neurite growth (GrandPre et al., 2000; Oertle et al., 2003; Prinjha et al., 2000). The 66 amino acid region in the C-terminal domain (Nogo66), also common to other Nogo splice variants, i.e. Nogo B and C, binds to the Nogo66 receptor NgR (Barton et al., 2003; Fournier et al., 2001; He et al., 2003). The Nogo66 signaling complex involves NgR, p75/Troy, LINGO-1 and, in some types of neurons, PirB (Atwal et al., 2008; Fournier et al., 2001; Mi et al., 2004; Wong et al., 2002). This signaling complex can also be activated by other myelin inhibitory proteins such as myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) (David and Lacroix, 2003; Filbin, 2003; Yiu and He, 2003). The known signaling components are summarized in the Figure 2.

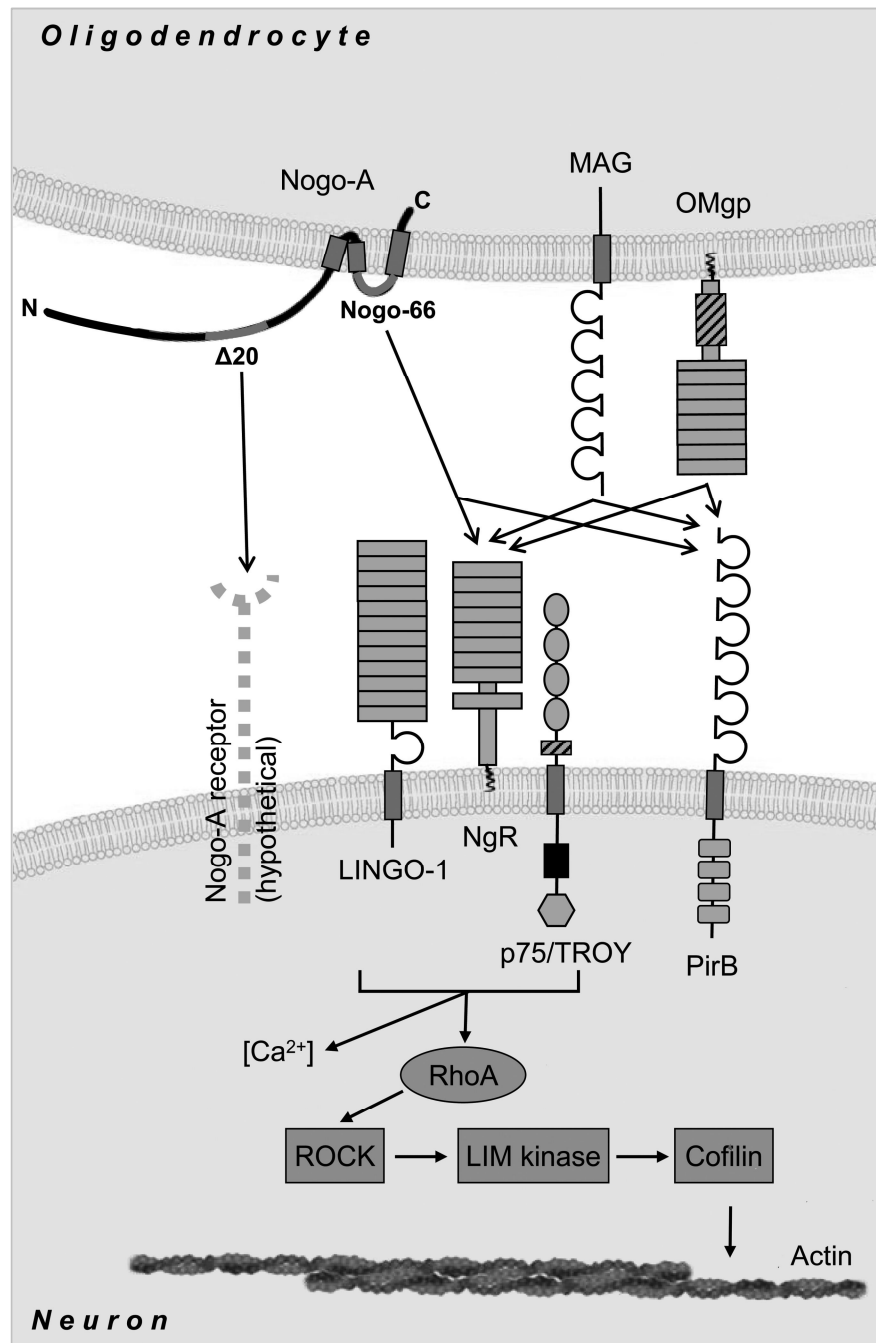


Figure 2: Nogo-A downstream signals, adapted from Schwab 2004

However, blocking NgR does not completely abolish myelin inhibition of neurite outgrowth suggesting the existence of an NgR independent mechanism. A 181 amino acid region in the central region of the Nogo-A (AA 544-725) protein called Nogo 20 is Nogo-A specific and is highly inhibitory for spreading and outgrowth of neurons and fibroblasts even in the absence of NgR (Oertle et al., 2003). The *in vivo* application of the monoclonal antibody 11C7, which is directed against this region and blocks Nogo 20 function, leads to enhanced growth and regenerative sprouting of spinal axons following spinal cord lesion in rats and monkeys (Freund et al., 2006; Liebscher et al., 2005). *In vitro*, Nogo 20 induces growth cone collapse and activates the small GTPase Rho-A (Fournier et al., 2003; Niederost et al., 2002; Oertle et al., 2003). The molecular nature of the Nogo 20 high affinity binding component is currently under investigation.

1.2 Endocytosis

The plasma membrane is the interface between the extracellular and the intracellular milieu of a cell. The entry and exit of small and large molecules is highly regulated. Whereas small molecules cross the plasma membrane through channels and membrane pumps, macromolecules are transported in membrane-bound vesicles derived by invagination and pinching-off of the plasma membrane, a process termed endocytosis. Endocytosis can occur via various mechanisms, which can be divided into two main categories, phagocytosis also termed *cell eating* and pinocytosis also termed *cell drinking*. Phagocytosis is restricted to specialized mammalian cells like macrophages, neutrophils or dendritic cells, whereas pinocytosis is common to all cells. Pinocytosis can occur by different pathways: macropinocytosis, clathrin-dependent, caveolae-dependent and clathrin- and caveolae-independent pathways (Le Roy and Wrana, 2005; Mayor and Pagano, 2007). The known endocytic pathways are schematically shown in Figure 3.

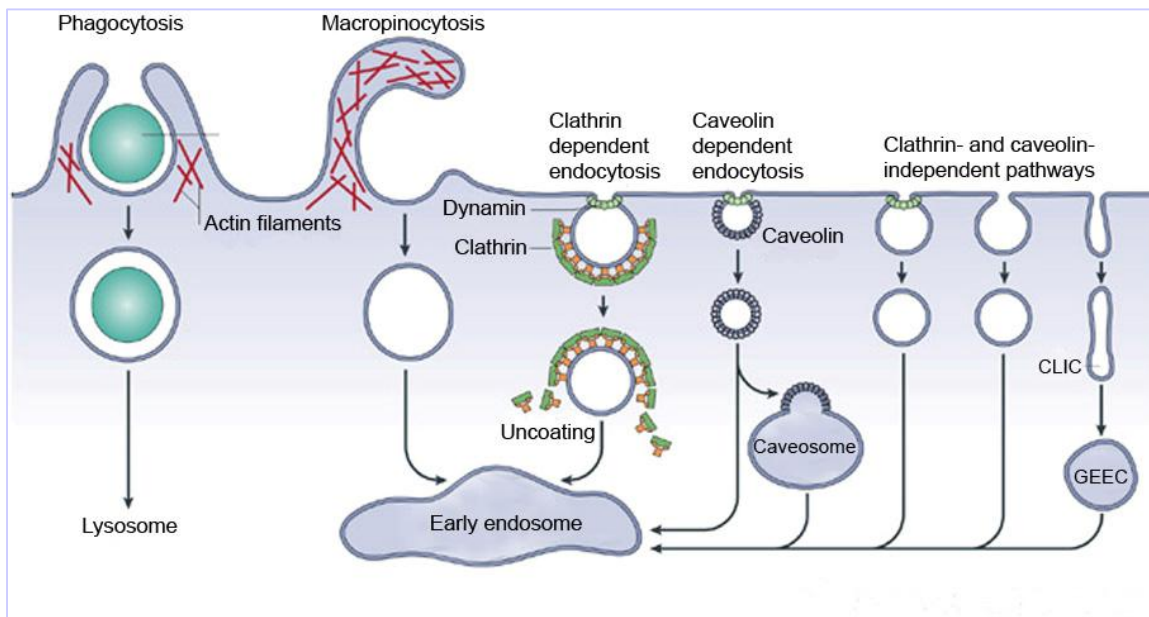


Figure 3: Schematic representation of different endocytic pathways with their intermediates: clathrin-coated vesicles, caveosomes, CLathrin-Independent Carriers (CLIC), and GPI-enriched endosomal compartments (GEEC) (Mayor and Pagano, 2007).

The evolutionary beginnings of the first primitive endocytic events are believed to have occurred during prokaryote-to-eukaryote transition. De Duve suggested that as early cells moved from the concentrated "primordial soup" to more dilute environment of nascent oceans, the existing strategy of external nutrient digestion with secreted exoenzymes became inadequate. Thus, the early cells had to convert from extracellular digestion to intracellular digestion by taking up extracellular materials and secreting digestive enzymes into these internalized vesicles. De Duve referred to this transition as "the beginning of cellular emancipation": *Until then, in order to benefit of their exoenzymes, the cells had to rely on extracellular digestion. Unless they had other means of subsistence, they were practically condemned to reside inside their food supply, like maggots in a chunk of cheese. Henceforth, they would be free to roam the world and to pursue their prey actively, living on phagocytized bacteria or on other engulfed materials. This development could well have heralded the beginning of cellular emancipation.* (De Duve R, 1991). The mechanisms of endocytosis have diversified considerably since the beginning of this "cellular emancipation". The number of molecules involved in the endocytic machinery is still growing. This complexity allows the cell to highly regulate its cellular trafficking thereby controlling crucial cellular events such as development, immune response, neurotransmission and signal transduction.

The most efficient uptake of macromolecules is during receptor-mediated endocytosis. Upon binding of ligands to their cell surface receptors, receptor-ligand complexes are recruited into coated pits. The process begins by assembling plasma membrane proteins and lipids through interactions with cytosolic adaptors and accessory proteins. As the endocytic vesicle forms, the plasma membrane with the associated proteins is pulled into the cytosol. Upon scission the coated vesicle is released into the cytoplasm (Figure 4). In the cytoplasm, the endocytic vesicles become uncoated and fuse with specialized membrane organelles known as early endosomes. The early endosomal compartment acts as the main sorting station in the endocytic pathway. Internalized receptor-ligand complexes can undergo different fates. Most ligands dissociate from their receptors in the acidic environment of the early endosomes. The receptors can either be

recycled back to the plasma membrane or directed to the lysosomal compartment, where they will be degraded (Alberts et al., 2002).

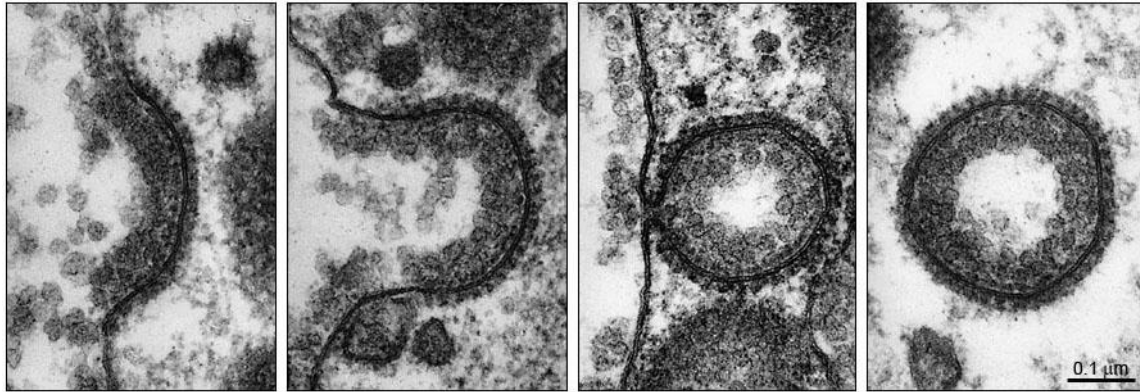


Figure 4: Vesicle formation observed by electron microscopy (McMahon and Gallop, 2005).

Clathrin-dependent pathway

Clathrin-coated vesicles are the best characterized endocytic vesicles (Figure 6). As the name indicates, the main component of the coat is clathrin. It has a three-legged structure, called a triskelion, consisting of three large and three small polypeptide chains (Figure 5) (Nathke et al., 1992). The adaptor proteins (AP) such as AP2 (Kirchhausen, 1999), epsin (Chen et al., 1998) and AP180 (Ford et al., 2001) bind to clathrin and stimulate its polymerization into pentagons and hexagons.

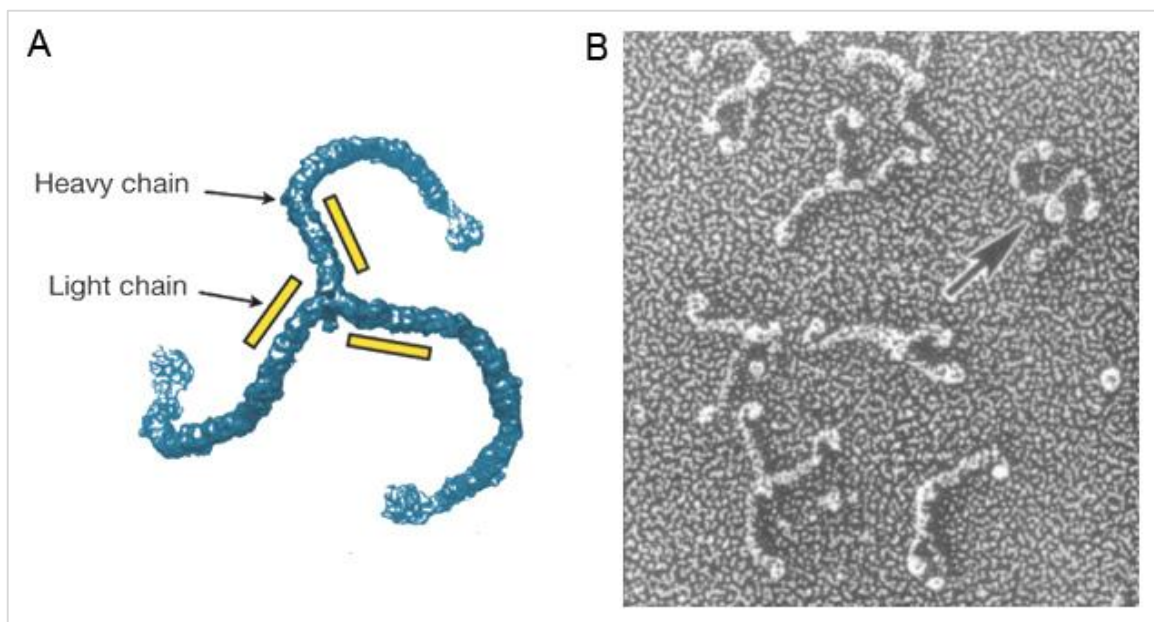


Figure 5 Clathrin Triskelion: (A) Schematic representation of clathrin chains (Fotin et al., 2004). (B) First electron micrographs of clathrin triskelion (Kirchhausen et al., 1986).

Other accessory proteins such as endophilin and amphypysin assist by membrane deformation. Both proteins form amphipathic helices, which may intercalate into one leaflet of the bilayer thereby increasing the membrane curvature (Itoh and De Camilli, 2006). The GTPase dynamin act as mechanochemical molecular spring by catalyzing the fission of nascent clathrin-coated vesicles from the plasma membrane (Stowell et al., 1999). Clathrin-mediated endocytosis occurs in all mammalian cells and is implicated in the continuous uptake of essential nutrients, such as cholesterol in the form of low-density lipoprotein (LDL) and iron bound to transferrin.

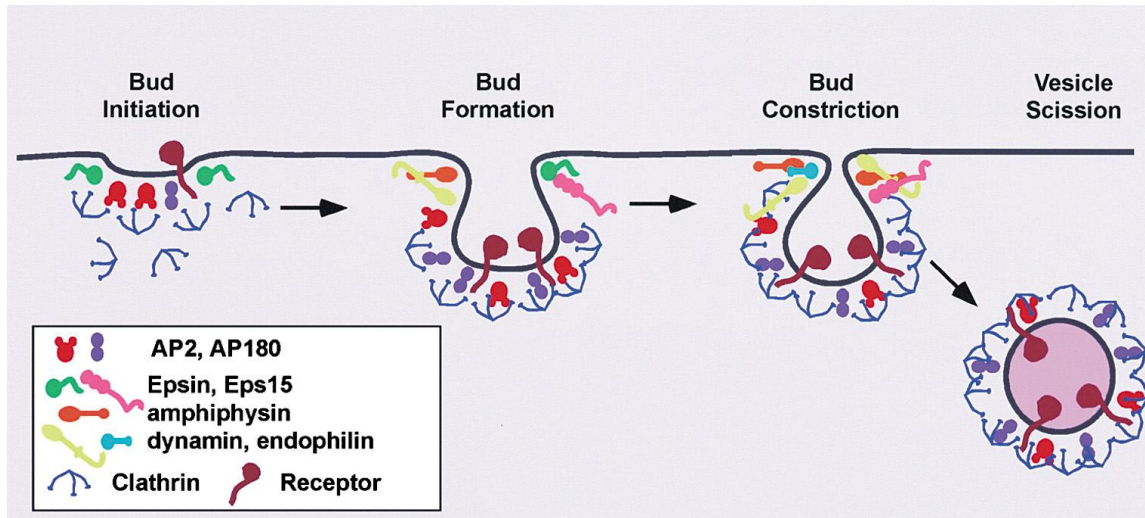


Figure 6 Accessory proteins during clathrin coat formation and scission (Hurley and Wendland, 2002)

Caveolin-dependent pathway

The plasma membrane of most cells has flask-shaped invaginations enriched in cholesterol and sphingolipids called caveolae (Rothberg et al., 1992). The best characterized component of caveolae is caveolin, a dimeric protein that binds cholesterol (Rothberg et al., 1992). Three caveolin proteins exist in mammals but their expression varies considerably between tissues. The highest levels of caveolin-1 are found in adipocytes, endothelia, pneumocytes and smooth muscle cells. Caveolin-2 is co-localized and co-expressed with caveolin-1. However, caveolin-3 is mainly expressed in muscle cells (Williams and Lisanti, 2004). The polymerase 1 transcript release factor (PTRF), also known as cavin-1 has been identified as an additional caveolar component required for caveola formation (Aboulaich et al., 2004; Hill et al., 2008; Liu et al., 2008; Liu and Pilch, 2008). Recently, a third protein, serum deprivation protein response (SDPR), also known as cavin-2, has been implicated in the formation of caveolar invagination. SDPR binds to PTRF and promotes its recruitment to caveolae (Hansen et al., 2009). However, how SDPR, PTRF and caveolin induce membrane curvature remains still unknown (Hansen et

al., 2009). Additionally, another PTRF homologue, SRBC, also known as cavin-3, has also been shown to regulate caveolae endocytosis (McMahon et al., 2009). Since numerous signaling molecules are associated with caveolae, it is believed that caveolin-dependent endocytosis has a role in regulation of specific signaling cascades (Anderson, 1998; Razani et al., 2002).

Studies with simian virus 40 (SV 40) as a marker for caveolin-dependent uptake led to characterization of caveolin-1 positive endosomes, called caveosomes (Pelkmans et al., 2001). This internalization requires cholesterol, dynamin, Src-family kinases and local actin polymerization (Pelkmans et al., 2002; Sverdlov et al., 2007).

GPI-enriched endosomal compartments (GEEC) pathway

Caveolae represent just one type of cholesterol-rich microdomains. Glycosyl-phosphatidylinositol (GPI) anchored proteins (GPI-APs), e.g. are also organized in cholesterol rich microdomains at the cell surface (Varma and Mayor, 1998). GPI-APs can be internalized into CLathrin-Independent Carriers (CLICs; Figure 1.3,1.7) which fuse to form tubular early endosomal compartment called GPI-AP-Enriched early Endosomal Compartments (GEECs) (Kirkham et al., 2005; Sabharanjak et al., 2002). Current knowledge about the molecular mechanisms underlying the GEEC pathway is very limited. Recently, the protein GTPase Regulator Associated with Focal adhesion kinase-1 (GRAF1) has been identified as the first specific component of CLICs and is thought to be involved in the membrane deformation and scission (Lundmark et al., 2008). In addition, it has been shown that CLIC formation is dynamin-independent and requires cholesterol-sensitive Cdc42 activation (Chadda et al., 2007; Sabharanjak et al., 2002). The activity of Cdc42 is controlled by an Arf1-dependent recruitment of RhoGAP domain-containing protein, ARHGAP10 (Kumari and Mayor, 2008).

Flotillin-dependent pathway

Another type of clathrin-independent endocytosis involving caveolar structures is the flotillin-dependent endocytosis (Figure 7). Flotillin, also called Reggie proteins, are considered to belong to the SPFH (Stomatin/ Prohibitin/ Flotillin/ Hflk/C) protein superfamily. Two flotillin proteins exist: flotillin-1 (= reggie 2) and flotillin-2 (= Reggie 1) (Langhorst et al., 2005). They can associate to form homo- and heterooligomers (Frick et al., 2007). Flotillin microdomains form endocytic structures that are involved in the uptake of GPI-anchored proteins and glycosphingolipids (Blanchet et al., 2008; Frick et al., 2007; Glebov et al., 2006) similar to the caveolin-dependent and GEEC pathway. However, flotillin microdomains are believed to be separated from regions of the plasma membrane involved in the previously mentioned endocytic mechanism (Blanchet et al., 2008; Frick et al., 2007; Glebov et al., 2006). Flotillin internalization from the plasma membrane is controlled by the Fyn kinase, a Src-family kinase. Flotillins have been associated with many physiological processes such as phagocytosis, T-cell signaling, cell adhesion, regulation of actin cytoskeleton, and also with neurite growth and regeneration (Babuke and Tikkanen, 2007; Munderloh et al., 2009).

Macropinocytosis

Macropinocytosis refers to the generation of large endocytic vesicles (up to 5 μ m in diameter) and is accompanied by the formation of actin-dependent membrane ruffles (Figure 7). This process is induced in many cell types upon strong stimulation by growth factors such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) and is driven by Rac1 and Src kinases (Liberali et al., 2008; Ridley, 2001; Swanson and Watts, 1995). Internalization of major histocompatibility complex (MHC) proteins in dendritic cells runs via such a formation of large endosomal structures (Falcone et al., 2006; Garrett et al., 2000). Fission of nascent macropinosomes does not involve dynamin. As an alternative to dynamin, C-terminal binding protein 1 [CtBP1; also called brefeldin A-dependent ADP-ribosylation substrate (BARS)] (Corda et al., 2006; Weigert et al., 1999) has been proposed to function during membrane fission (Bonazzi et al., 2005; Hidalgo Carcedo et al., 2004). It has been shown that endocytosis of

human adenovirus requires CtBP1 (Amstutz et al., 2008). CtBP1 is locally recruited to the closure site of a macropinocytic cup where it is phosphorylated by p21-activated kinase-1 (PAK1) (Bokoch, 2003; Liberali et al., 2008). PAK1 itself was shown to be activated by small GTPases Rac1 and Cdc42 (Dharmawardhane et al., 1997; Zhang et al., 1995) and required for the uptake of the picornavirus echovirus 1 (Karjalainen et al., 2008). Therefore macropinocytosis can be described as a clathrin- and dynamin-independent but PAK1- and CtBP1-dependent endocytic process.

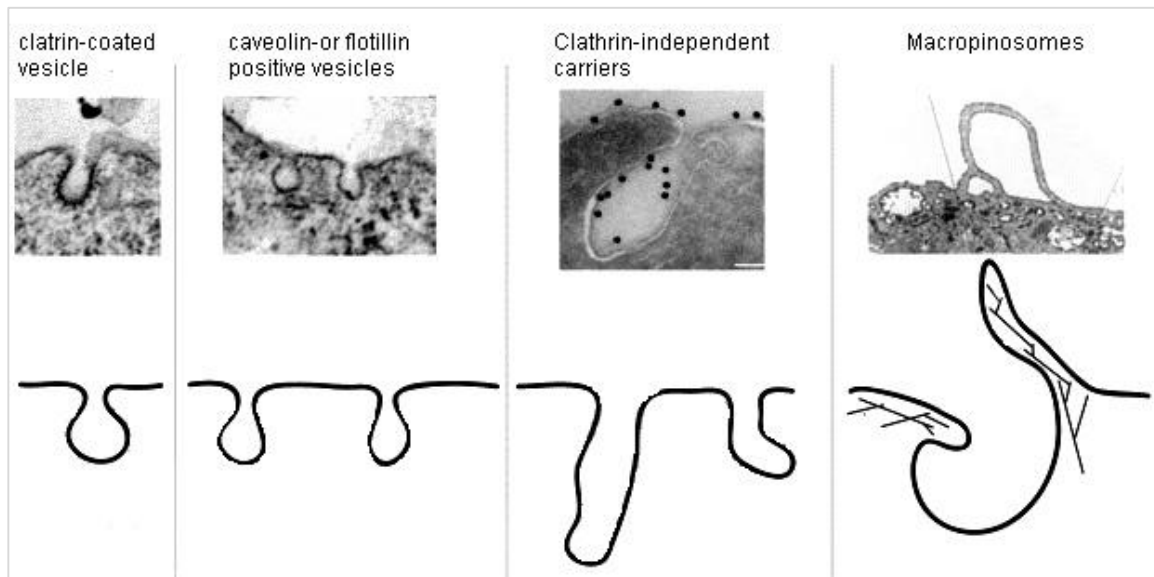


Figure 7: Morphological structure of different endocytic vesicles (Hansen and Nichols, 2009)

Pincher-dependent macroendocytosis

Pincher-dependent endocytosis involves generation of large endosomal structures, which are characterized by the presence of the PINocytic CHaPeron (Pincher) protein. Pincher belongs to the family of Eps15 homology (EH)-domain-containing proteins (EHDs/RME-1) which have been implicated in recycling from endosomes (Caplan et al., 2002; Grant et al., 2001). Pincher-dependent macroendocytosis has been shown to mediate internalization of Trks (tropomyosin-

related kinases), the neurotrophin receptors, into neurons. This process depends on Rac but not on clathrin. The formation of Trk containing vesicles is required for proper retrograde signaling in both peripheral and central neurons (Shao et al., 2002; Valdez et al., 2007). Pincher has recently been classified as a member of the dynamin superfamily and as a ATPase which may play a role during vesicle fission (Daumke et al., 2007).

Endocytosis and Signaling

For a long time it has been believed that endocytosis serves only for signal termination by down-regulation of the cell surface receptors. This view was challenged with the observation that the endosomes containing internalized epidermal growth factor receptor (EGFR) also contained downstream signaling factors such as SHC (SH2-domain-containing transforming protein), Grb2, mSOS (mammalian son-of sevenless) and MAPK (mitogen-activated protein kinase) (Di Guglielmo et al., 1994; Vieira et al., 1996). Subsequent experiments showed that signals from EGFR-containing endosomes are sufficient to promote MAPK activation and cell survival (Wang et al., 2002c). This concept of endosomal signaling can generally be applied to other receptor tyrosine kinases (RTKs). The activation of PDGF (platelet-derived growth factor) receptors in the endosomal compartments can initiate recruitment of downstream signaling components and promote cell survival by up to 50% (Wang et al., 2004). Similar results have been obtained for TrkB upon stimulation with BDNF and for TrkA upon stimulation with NGF. Inhibition of TrkA and TrkB endocytosis leads to enhanced neuronal cell death (Heerssen et al., 2004; Ye et al., 2003). In addition TrkB endocytosis is also required for directed cell migration (Zhou et al., 2007).

Another important role of endocytosis has been shown during development. The internalization of the Notch receptor and its ligands and the subsequent endosomal sorting is required for asymmetric cell division (Coumailleau et al., 2009; Le Borgne et al., 2005; Seugnet et al., 1997). Endocytic trafficking of cell adhesion molecules such as E-cadherin regulates epithelial polarity and cell migration (Emery and Knoblich, 2006; Lu and Bilder, 2005; Ulrich et al., 2005).

Inhibition of ephrin-eph endocytosis leads to abnormal retinogeniculate axonal projections during development (Cowan et al., 2005).

Retrograde Axonal Transport

In the nervous system, endocytosis plays a crucial role for long-range communication within neurons. Neurons send their axons over long distances to contact post-synaptic targets. In the human nervous system these distances can be more than 1 meter. Signals generated at the axon terminals have to be transmitted from the nerve terminal to the cell bodies to activate gene transcription, a mechanism termed retrograde signaling (Howe and Mobley, 2005; Ibanez, 2007; Keshishian and Kim, 2004). For short distances retrograde signals are transmitted by the propagation of the Ca^{2+} waves. Application of Slit-2 to the leading tip of a migrating cerebellar granule neuron e.g., evokes a Ca^{2+} wave, which is propagated from the growth cone to the soma leading to growth cone collapse and reversal of neuronal migration (Guan et al., 2007).

One possible way of long distance retrograde signaling is the axonal retrograde transport of the ligand-receptor complexes or second messenger complexes. This has been extensively studied for the neurotrophins such as BDNF and NGF (Campenot and MacInnis, 2004; Howe and Mobley, 2005; Huang and Reichardt, 2001; Wu et al., 2009). NGF was discovered as the first growth factor required for the survival of the peripheral neurons (Cohen and Levi-Montalcini, 1956). It is secreted by target tissues such as skin, muscle, glands or neurons (Levi-Montalcini, 1987; Thoenen, 1991). Upon the release it binds to the TrkA receptor on the axonal terminals. The activation of the TrkA receptor leads to the recruitment of downstream signaling components such as phospho-ERK5, phospho MEK1, phospho-MAPK p38, B-Raf, Gab2 and Rap1 into EEA-1 and Rab-5 positive endosomes (Delcroix et al., 2003). The NGF signaling endosome, also called NGF signalosome then associates with dynein and is retrogradely transported in a microtubule-dependent manner (Yano et al., 2001). Upon arrival in the cell bodies NGF signalosomes activate CREB-dependent (Cox et al., 2008; Riccio et al., 1997; Watson et al.,

1999) and SRF-dependent (Wickramasinghe et al., 2008) gene transcription (Figure 8). The generation of NGF signalosome is mediated by the Pincher protein (Valdez et al., 2005).

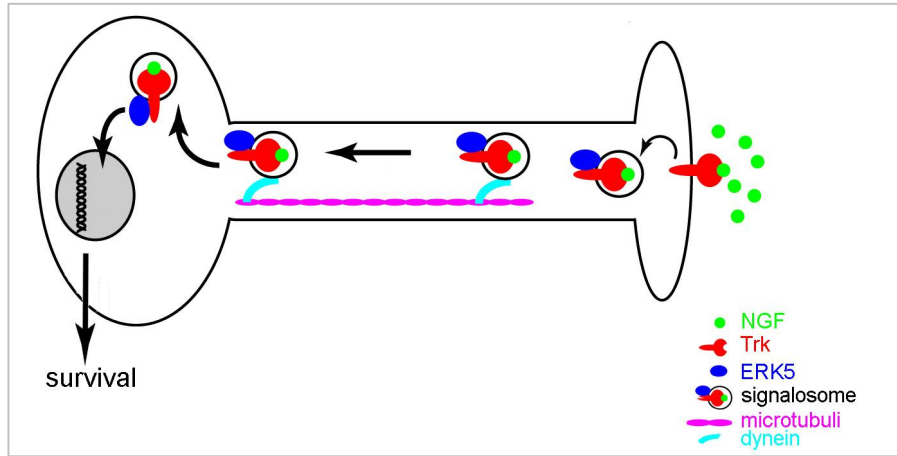


Figure 8: Retrograde axonal transport of the NGF signalosome (Howe, 2005)

Context and Aim of the Thesis

In the past years, the role of Nogo-A as a potent inhibitor of axon growth and plasticity in both, injured and intact adult mammalian CNS has been well documented. In addition, novel roles for Nogo-A as a regulator of fasciculation, axon pathfinding and migration in both the PNS and CNS are emerging. Whereas the signaling mechanisms of the C-terminal inhibitory domain of Nogo-A, the Nogo66 region, are well characterized, comparatively little is known about the signaling complex of the Nogo-A specific N-terminal inhibitory region, Nogo 20. Since blocking of the NgR complex does not completely abolish myelin inhibition of neurite outgrowth, it is of great importance to elucidate the NgR independent mechanism of Nogo-A signaling. The following chapter aimed to characterize cellular and molecular events evoked after Nogo 20 addition to the cell surface of responsive cells. Many surface ligand-receptor complexes are known to internalize into cells and signal from endosomes. The endosomal signaling has been well studied in the context of the neurotrophic factors such as NGF. The formation of NGF signalosomes is necessary for survival of peripheral neurons. Not only growth cone attracting molecules such as NGF, also growth cone repulsive molecules such as ephrins have been shown to depend on internalization for their signaling. Inhibition of ephrin endocytosis abolishes ephrin-induced growth cone collapse and leads to abnormal retinogeniculate projections. Therefore, we addressed whether endocytosis contributes to Nogo 20 signaling in the neuronal cells. Similar to the neurotrophic factors, Nogo-A provokes short-term effects locally at the growth cone, but also long-term effects by inducing persistent axon outgrowth effects. Since the members of the neurotrophin family induce changes in gene transcription in the cell body upon retrograde axonal transport leading to cell survival, we asked whether Nogo 20 also induces formation of signaling endosomes, which are retrogradely transported in order to activate a neurite outgrowth inhibition response in the cell body. Although the receptor for Nogo 20 has not yet been identified, the findings presented in the next chapter should shed more light on the Nogo 20 specific mechanism.

õAll truths are easy to understand once they are discovered; the point is to discover them.õ

Gallileo Gallilei

Chapter 2

Pincher-generated Nogo-A endosomes mediate growth cone collapse and retrograde signaling

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Contributions as author: I have designed and performed all the experiments, analyzed the data, and written the manuscript.

Abstract

Nogo-A is one of the most potent myelin-associated inhibitors for axonal growth, regeneration and plasticity in the adult central nervous system. The Nogo-A specific fragment Nogo 20 shows the most potent inhibitory effect on growth cone collapse, neurite outgrowth and cell spreading by activating RhoA. Here we show that Nogo 20 is internalized into neuronal cells by a Pincher- and rac-dependent, but clathrin- and dynamin-independent mechanism. Pincher-mediated macroendocytosis results in the formation of Nogo 20 signalosomes that direct RhoA activation and growth cone collapse. In compartmentalized chamber cultures, Nogo 20 is endocytosed into neurites and retrogradely transported to the cell bodies of DRG neurons, triggering RhoA activation en route and decreasing pCREB levels in cell bodies. Thus, Pincher-dependent macroendocytosis may lead to the formation of a Nogo-A signaling endosome, which acts both within growth cones and after retrograde transport in the cell body to regulate the neuronal growth program.

Introduction

One of the most potent neurite growth inhibitors of the adult central nervous system (CNS) is the transmembrane protein Nogo-A (Cafferty and Strittmatter, 2006; Schwab, 2004; Yiu and He, 2006). The suppression of Nogo-A signaling by either Nogo-A neutralization or blockade of the Nogo66 receptor (NgR) components or inhibition of downstream signaling components such as Rho-A and Rho-A kinase (ROCK) leads to enhanced regeneration and nerve fibre growth associated with increased functional recovery in the adult CNS after injury (Cafferty and Strittmatter, 2006; Schwab, 2004; Yiu and He, 2006). Besides its role in the injured mammalian CNS, Nogo-A acts as a regulator of neuronal growth and plasticity in the intact CNS. For instance, the plasticity of the visual cortex is extended beyond the normal postnatal critical period in mice lacking NgR or Nogo-A/B (McGee et al., 2005). In the intact adult spinal cord and cortex genetic ablation of Nogo-A resulted in an enhanced expression of many proteins involved in neuronal growth and cytoskeletal organization in the neurons and growth cones (Montani et al., 2009).

Nogo-A is a large membrane protein of 1163 amino acids containing two main inhibitory regions for neurite growth (GrandPre et al., 2000; Oertle et al., 2003; Prinjha et al., 2000). The 66 amino acids region in the C-terminal domain (Nogo66), also common to other Nogo splice variants, i.e. Nogo B and C, binds to the Nogo66 receptor NgR (Barton et al., 2003; Fournier et al., 2001; He et al., 2003). The Nogo66 signaling complex involves NgR, p75/Troy, LINGO-1 and, at least in some types of neurons, PirB (Atwal et al., 2008; Fournier et al., 2001; Mi et al., 2004; Wong et al., 2002). This signaling complex can also be activated by other myelin inhibitory proteins like myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) (David and Lacroix, 2003; Filbin, 2003; Yiu and He, 2003). However, blocking NgR does not completely abolish myelin inhibition of neurite outgrowth suggesting the existence of an NgR independent mechanism (Kim et al., 2004). A 181 amino acid region in the central region of the Nogo-A protein called Nogo 20 is Nogo-A specific and is highly inhibitory for spreading and outgrowth of neurons and fibroblast even in the absence of NgR (Oertle et al., 2003). The *in vivo*

application of the monoclonal antibody 11C7, which is directed against this region and blocks Nogo 20 function, leads to enhanced regrowth and regenerative sprouting of spinal axons following spinal cord lesion in rats and monkeys (Freund et al., 2006; Liebscher et al., 2005). *In vitro*, Nogo 20 induces growth cone collapse and activates the small GTPase Rho-A (Fournier et al., 2003; Niederost et al., 2002; Oertle et al., 2003). However, the molecular mechanisms underlying Nogo 20 signaling remain mostly unknown.

Similar to the neurotrophic factors including nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurotrophin 3 or 4 (NT-3 and NT-4) (Campenot and MacInnis, 2004; Huang and Reichardt, 2001; Wu et al., 2009), Nogo-A acts locally, at the growth cone. In addition, the members of neurotrophin family induce changes in gene transcription in the cell body upon retrograde axonal transport (Ginty and Segal, 2002; Howe and Mobley, 2005). Detailed analysis of NGF retrograde signaling led to the characterization of a so called NGF "signalosome", a signaling endosome containing endocytosed ligand-receptor complexes and downstream effectors (Campenot and MacInnis, 2004; Ginty and Segal, 2002; Howe and Mobley, 2005). Up to now, the possible role of endocytic signaling as a mechanism for Nogo-A action, both locally and at the level of cell body has not been investigated.

Here, we show that Nogo 20 actions on growth cone collapse require signaling from endosomes that contain activated Rho. Internalization into the signaling endosomes is clathrin-independent and occurs by Pincher-dependent endocytosis. The retrograde axonal transport of Nogo 20 in DRG neurons results in increased Rho-GTP and decreased levels of pCREB in the soma.

Results

The Nogo-A active fragment Nogo 20 is rapidly internalized into neurons

To examine whether the Nogo-A active fragment Nogo 20 is internalized into Nogo-A responsive cells, PC12 neuron-like cells were incubated with 300 nM T7-tagged Nogo 20 fragment (Oertle et al., 2003). As control, we used 300 nM Nogo 21-T7, a Nogo-A fragment without inhibitory activity at this concentration (Oertle et al., 2003). First, we incubated PC12 cells with Nogo fragments for 1h at 4°C, a temperature which prevents endocytosis and vesicular trafficking. PC12 cells immunostained for the T7 tag and analyzed by confocal microscopy displayed patchy staining at the cell surface (Figure 1A). When the PC12 cells were incubated with the Nogo 20 fragment for 15 (Figure 1B) or 30 min (Figure 1C) at 37°C, the tagged protein was massively endocytosed in small cytosolic vesicles. The control fragment Nogo 21-T7 could not be detected, either on the cell surface or intracellularly after incubation of PC12 cells (data not shown). The early endosomal antigen EEA-1, a marker of the early endosome (Mu et al., 1995), co-localized with 40.57 ± 2.65 % (n = 47 cells) of Nogo 20-T7 positive vesicles after 15 min (Figure 1B; 1F) and with 57.87 ± 3.99 % (n = 42 cells) after 30 min (Figure 1C, 1F). The internalization of Nogo 20 was further characterized by subcellular fractionation. PC12 cells were incubated with 300 nM Nogo 20 for 30 min at 37°C, lysed and subjected to a 8-40.6% sucrose step gradient centrifugation. Nogo 20 was detected in the fractions with lower sucrose concentration, F1 (8-25%) and F2 (25-35%), which contained early endosomes as indicated by EEA1 (Figure 1D). In contrast, Nogo 20 was absent from the nuclear fraction (NF; containing nucleoporin p62 and from fraction F3 containing mostly endoplasmatic reticulum and Golgi membranes (high sucrose concentration of 35-40.6%; Figure 1D).

The fate of the growth inhibitory Nogo-A fragment Nogo 20 was also studied in hippocampal neurons dissected from E19 rats, cultured for 4 days. Incubation with 300 nM Nogo 20-T7 for 30 min at 37°C resulted in many small fluorescent vesicles spread throughout the cytoplasm of cell bodies and neurites (Figure 1E). These findings show that upon surface binding Nogo- 20 is rapidly internalized into neuronal endosomes.

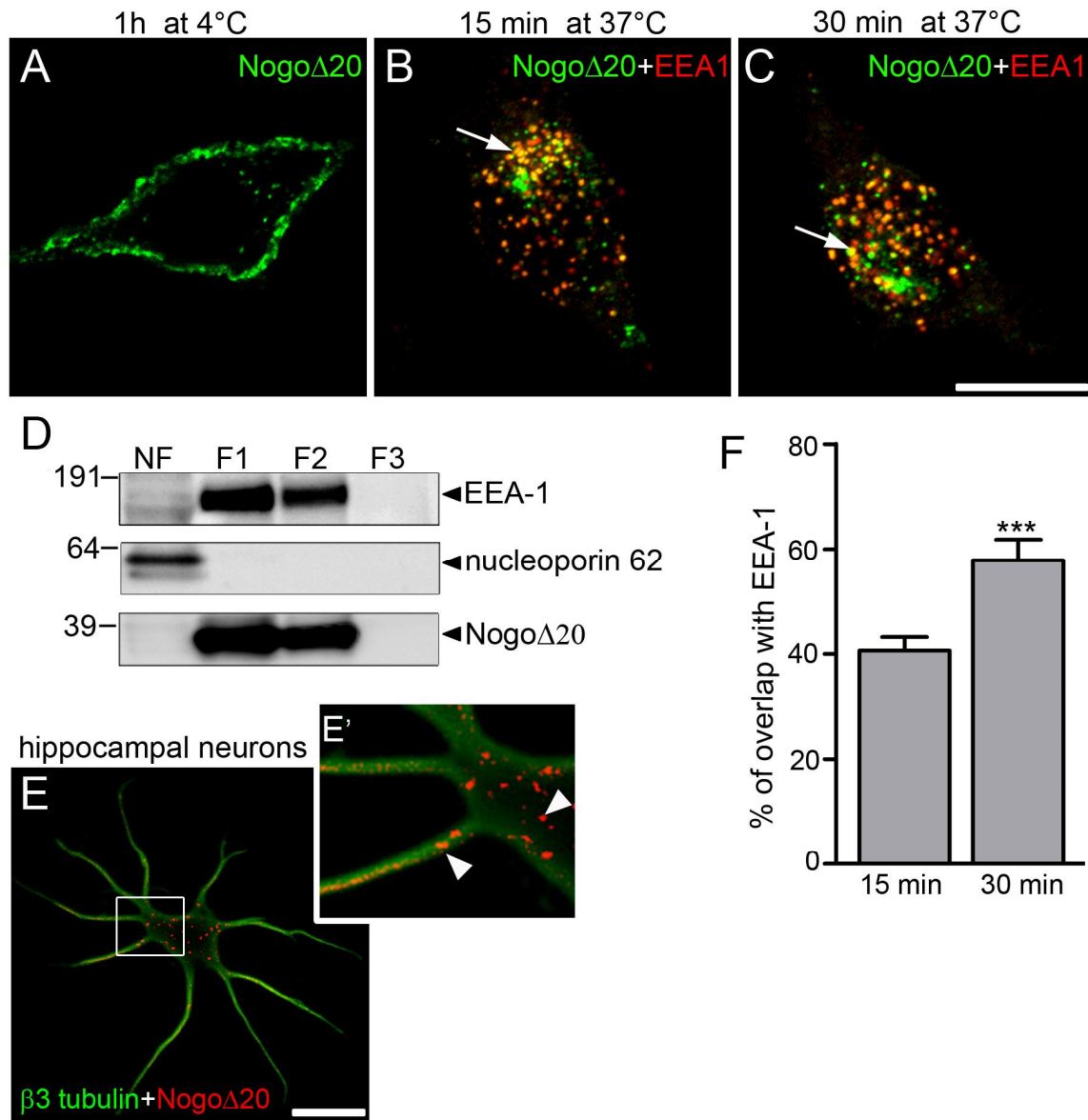
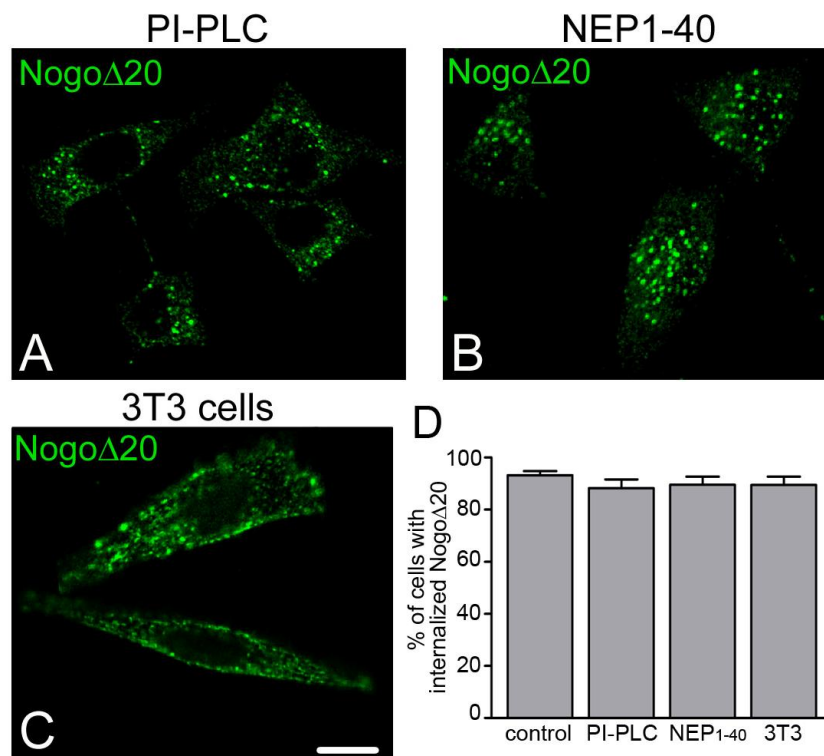


Figure 1. Surface binding and internalization of Nogo 20 in neuronal cells (A) ó (C): Representative confocal immunofluorescence optical sections of PC12 cells which were incubated with 300 nM Nogo 20-T7 for 1 hour at 4°C (A) or for 15 min (B) or 30 min (C) at 37°C. Cells were stained with anti-T7 mAb for Nogo 20 (green) and with anti-EEA-1 as an early endosomal marker (red) and analyzed by confocal microscopy. Arrows indicate colocalization of Nogo 20 with EEA-1. Scale bar, 10 µm. (D) Subcellular fractionation of PC12 cells after internalization of Nogo 20-T7 for 30 min at 37°C. Cells were homogenized and centrifuged to separate nuclei (nuclear fraction NF) from the postnuclear supernatant, which was loaded on a 8-40.6% sucrose step gradient to separate different organelles. Following centrifugation, different fractions: F1 (8-25% sucrose), F2 (25-35% sucrose) and F3 (35-40.6% sucrose) were collected and immunoblotted for EEA-1 (top panel), nucleoporin p62 (middle panel) and Nogo 20-T7 (bottom). A representative blot from 3 experiments is shown. (E) Dissociated hippocampal neurons cultured for 4 days were incubated with 300 nM Nogo 20-T7 for 30 min at 37°. Nogo 20 (red) appears in vesicular structures in the cell body and neurites of the neurons (3 tubulin, green). Scale bar, 10 µm. (F) Quantification of co-localization of Nogo 20 with EEA-1 after indicated time points. Values are given as the mean from three independent experiments ± SEM (**P < 0.01, Student's *t* test).

To investigate a possible contribution of the Nogo66 receptor subunit NgR to the Nogo 20 internalization, PC12 cells were treated with phosphatidylinositol-specific phospholipase C (PI-PLC) to remove GPI-anchored proteins including NgR from the cell surface (Fournier et al., 2001) or alternatively, with the peptide, NEP1640, an NgR antagonist (GrandPre et al., 2002). Neither PI-PLC (Supplementary Figure 1A, 1D) nor NEP1640 (Supplementary Figure 1B, 1D) treatment affected Nogo 20 internalization. In addition, we also observed Nogo 20 internalization into 3T3 cells a cell line which does not express NgR (Fournier et al., 2001; Oertle et al., 2003) (Supplementary Figure 1C, 1D). These data demonstrate that Nogo 20 internalization is not dependent on the Nogo66 receptor NgR.



Supplementary Figure 1. Nogo 20 internalization does not depend on NgR

(A),(B): Representative confocal immunofluorescence optical sections of PC12 cells which were pretreated with PI-PLC (A) or NEP1-40 (B) one hour prior to incubation with 300 nM Nogo Δ 20-T7 for 30 min at 37°C. Nogo 20 positive vesicles (green) were observed. (C) Nogo 20 (green) is internalized into 3T3 cells after 30 min incubation time. Scale bar, 10 μ m. (D) (Quantification of Nogo 20 uptake after various cell treatments. The percentage of cells with internalized protein is given as the mean from three independent experiments \pm SEM.

Nogo 20 internalization does not follow the conventional endocytic routes

To test whether Nogo 20-T7 is internalized via one of the classical endocytic routes mediated by clathrin, caveolin or cholesterol, pharmacological inhibitors and dominant negative (dn) constructs were used (Figure 2A).

Eps 15 is crucial for clathrin-coated pit assembly; overexpression of dominant negative eps 15 blocks clathrin-dependent internalization (Benmerah et al., 1998). Transfection of PC12 cells with the dominant negative Eps 15 E 95/295 did not inhibit internalization of Nogo 20-T7 (94.58 ± 5.84 vs. 99.93 ± 7.61 in control, $n = 294$ vs. 323 cells) (Figure 2B, 2E). In contrast to Nogo 20, internalization of transferrin, a well established marker for clathrin-mediated uptake (Dautry-Varsat et al., 1983; Hopkins, 1983), was dramatically reduced ($9.44\% \pm 2.33$ vs. $99.88 \pm 5.75\%$ in control, $n = 279$ vs. 216 cells, $***P < 0.001$) (Figure 2B, 2E). These results show that eps15-mediated clathrin assembly is not essential for Nogo 20-T7 endocytosis.

The small GTPase Dynamin II is involved in the formation of both clathrin-coated and caveolar vesicles (De Camilli et al., 1995; Oh et al., 1998; Pelkmans et al., 2002). Surprisingly, the expression of a dominant negative dynamin II mutant, dynaminK44A (Fish et al., 2000), in PC-12 cells did not affect the internalization of Nogo 20-T7 ($90.12 \pm 2.95\%$ vs. $99.93 \pm 7.61\%$ in control, $n = 303$ vs. 323 in control) (Figure 2C, 2E). The internalization of transferrin, however, was almost completely inhibited ($4.86 \pm 1.67\%$ vs. $99.88 \pm 5.75\%$ in control, $n = 332$ vs. 216, $***P < 0.001$) (Figure 2C, 2E).

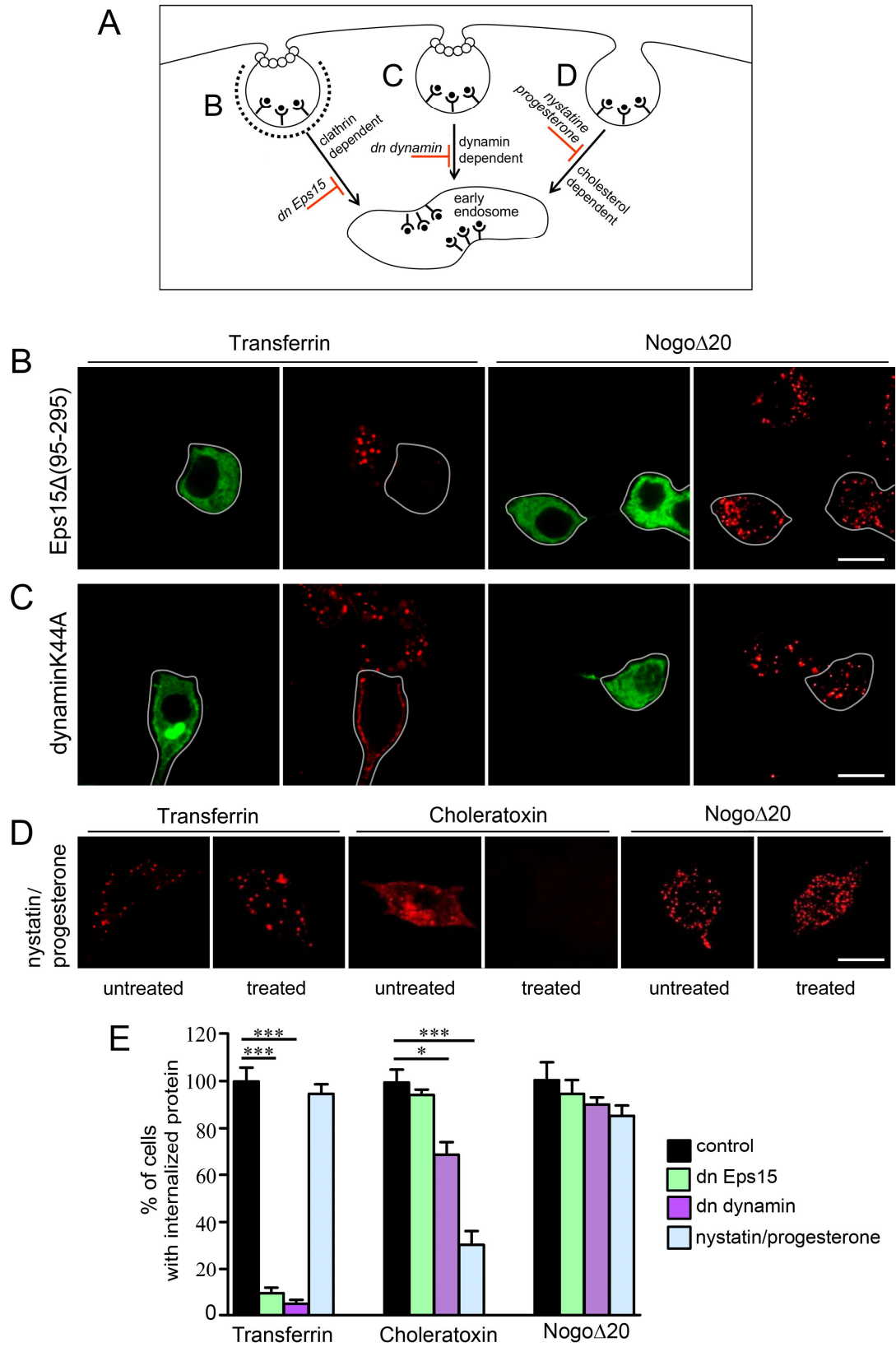


Figure 2

Figure 2. Internalization of Nogo 20 occurs independently of Epsin15, Dynamin II and cholesterol. (A) Schematic representation of different endocytotic pathways and their blockers. (B, C) PC12 cells were transfected with GFP tagged Eps15 (95-295) (green; B), or GFP-dynIIK44A (green; C). 24 hours later, cells were incubated with 300 nM Nogo 20-T7 (red) or 100 nM Transferrin-biotin (red) for 30 min at 37°C. In Eps15 (95-295) and dynIIK44A transfected cells Transferrin uptake was blocked, but not uptake of Nogo 20. (D) PC12 cells were either left untreated or were pretreated with nystatin and progesterone over night and were then incubated with Transferrin, Cholera toxin or Nogo 20-T7 for 30 min at 37°C in absence or presence of drugs. Whereas the internalization of Cholera toxin was inhibited (red), the internalization of Transferrin (red) and Nogo 20 (red) was not affected. Representative optical sections from three independent experiments are shown. Scale bar, 10 μ m. (E) Quantification of protein uptake after various cell treatments. The percentage of cells with internalized protein is given as the mean \pm SEM (* $P < 0.05$, *** $P < 0.001$, Student's t test).

Internalization via caveolae is cholesterol-dependent. The cholera toxin subunit (CTx) has been reported to be predominantly internalized through the cholesterol-sensitive pathway (Kirkham et al., 2005). To test the possibility that Nogo 20-T7 internalization might depend on cholesterol, we pretreated PC12 cells with nystatin and progesterone. Combined cholesterol depletion by nystatin and inhibition of cholesterol synthesis by progesterone resulted in a significant inhibition of the uptake of cholera toxin (30.33 ± 5.81 % vs. 99.40 ± 5.48 in control, $n = 478$ vs. 366 , *** $P < 0.001$) (Figure 2D, 2E) whereas Nogo 20-T7 endocytosis remained unaffected (85.32 ± 4.32 % vs. 99.93 ± 7.61 % in control, $n = 487$ vs. 323) (Figure 2D, 2E).

Taken together these data provide strong evidence that internalization of Nogo 20-T7 does not follow the conventional endocytic routes. Neither clathrin nor caveolin nor cholesterol were required for the internalization of Nogo 20-T7 into the PC12 cells.

Nogo 20 endocytosis is mediated by Pincher-dependent macroendocytosis

The PINocytic CHapERon protein Pincher belongs to the family of Eps15 homology (EH)-domain-containing proteins (EHDs/RME-1) which have been implicated in clathrin-independent endocytosis (Shao et al., 2002; Valdez et al., 2007) and recycling from endosomes (Caplan et al., 2002; Grant et al., 2001). Overexpression of a dominant negative form of Pincher (PincherG68E) has been shown to prevent NGF-induced internalization of TrkA (Shao et al., 2002). To assess a possible role of Pincher for Nogo 20-T7 endocytosis, we overexpressed dn HA-PincherG68E in PC12 cells. In agreement with previous observations (Shao et al., 2002), HA-PincherG68E was associated with the plasma membrane in PC12 cells. Interestingly, the expression of dominant-negative Pincher dramatically reduced the endocytosis of Nogo 20 (15.31 ± 3.68 % vs. 99.46 ± 5.49 % in control, $n = 47$ cells, $***P < 0.001$) (Figure 3A, 3F). Confocal analysis revealed that Nogo 20-T7 localization remained restricted to the plasma membrane and 73.37 ± 2.91 % of Nogo 20-T7 co-localized with PincherG68E (Figure 3B, 3E). In contrast, transferrin was internalized and appeared in a punctate pattern in the cytoplasm of the PC12 cells, consistent with previous results showing that PincherG68E does not interfere with clathrin-mediated endocytosis (Shao et al., 2002). Accordingly, we observed only 9.74 ± 2.02 % of transferrin overlap with PincherG68E (Figure 3B, 3E). These results indicate that Pincher function is essential for Nogo 20 endocytosis.

The small GTPase Rac has been shown to drive the formation of membrane ruffles during macroendocytosis and thus to be required for Pincher-mediated NGF-TrkA internalization (Valdez et al., 2007). To test whether Rac is involved in Nogo 20 internalization, we transfected PC12 cells with either wild-type Rac or the dominant-negative RacN17. The overexpression of RacN17 significantly blocked the internalization of Nogo 20 (26.08 ± 4.45 % vs. 99.46 ± 5.49 % in control, $n = 47$ cells, $***P < 0.001$). As shown by confocal microscopy, Nogo 20 remained at the plasma membrane of PC12 cells expressing RacN17 (Figure 3D, 3F). The overexpression of Rac1 had only a minor effect on the uptake of Nogo 20 (77.64 ± 5.12 % vs. 99.46 ± 5.49 % in control, $n = 47$ cells, $*P < 0.05$) (Figure 3D, 3F). These findings strongly suggest that Nogo 20 endocytosis is mediated by a macroendocytic process that depends on both Pincher and Rac proteins.

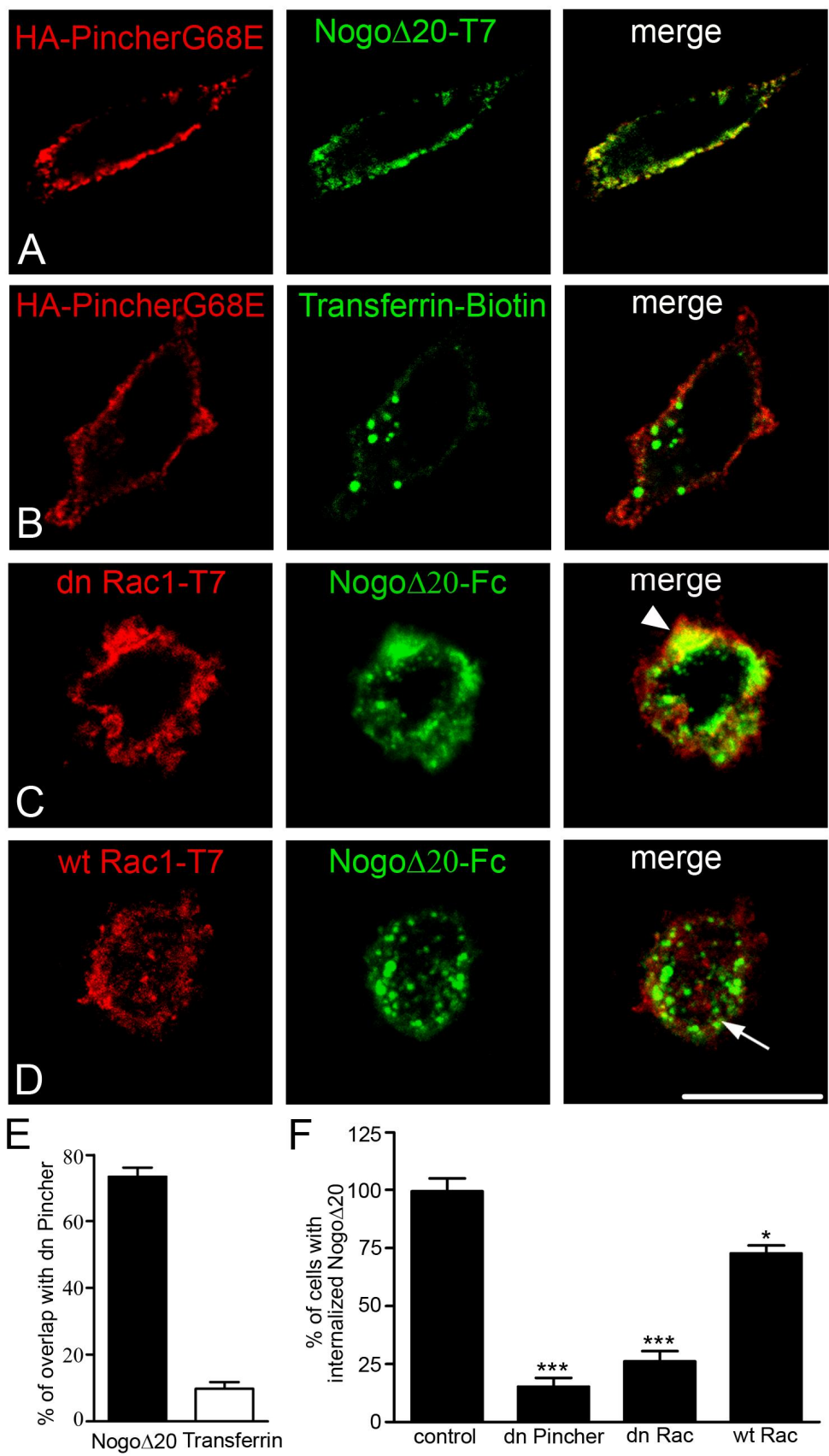


Figure 3. Internalization of Nogo 20 occurs by macroendocytosis and is Pincher and Rac1 dependent. (A) PC12 cells were transfected with HA tagged dn PincherG68E (red). 24 hours after transfection cells were incubated with 300 nM Nogo 20-T7 (green) for 30 min at 37°C. Nogo 20 remained on the cell surface; no internalized Nogo 20 vesicles are seen. (B) Transferrin (green) uptake is unaffected by overexpression of dn PincherG86E (red). (C, D) PC12 cells were transfected with T7 tagged wild-type Rac1 (red, C) or dominant negative Rac1N17 (red, D) prior to incubation with 300 nM Fc tagged Nogo 20 (green). After 30 min at 37°C Nogo 20 appeared a large number of endosomal vesicles and cisterns inside the wt Rac1 cells (arrowhead), but the internalized proportion was reduced in the dn RacN17 transfected cells (arrow). Scale bar, 5µm. (E) Quantification of Nogo 20 and transferrin co-localization with dn PincherG86E. Values are given as the mean of three independent experiments \pm SEM. (F) Quantification of internalization revealed that the uptake of Nogo 20 was reduced by 84 % upon overexpression of dn PincherG86E and by 73% upon overexpression of dn Rac1N17. Values are given as the mean from three independent experiments \pm SEM (*P < 0.05, ***P < 0.001, Student's *t* test).

Pincher-mediated Nogo 20 endocytosis is required in Nogo 20 induced growth cone collapse

Nogo 20 is a very potent inducer of growth cone collapse that often leads to withdrawal of neurites (Niederost et al., 2002; Oertle et al., 2003). To determine the importance of Nogo 20 endocytosis for the growth cone collapse, we examined the growth cone response of hippocampal neurons after blocking Nogo 20 endocytosis. Dissociated E19 hippocampal neurons were infected with recombinant adenoviruses containing either HA-Pincher or HA-PincherG68E (Valdez et al., 2005). Growth cone collapse was assessed at 4 DIV, i.e. 48 hours after viral infection by adding 300 nM of Nogo 20 to the medium for 30 min. The neurons were fixed and the morphology of the growth cones was visualized by staining for F-actin with Phalloidin-Alexa488 (Figure 4A and 4B). The infected neurons were identified by visualization of the HA tag (Figure 4C). Nogo 21 does not stimulate growth cone collapse at 300 nM, we thus used this fragment as a negative control. Three independent experiments (n=109 neurons) showed that Nogo 20 treatment induced collapse of 69.85 ± 2.55 % of the growth cones as compared to 25.11 ± 1.59 % observed after the Nogo 21 control treatment (Figure 4D). A very similar proportion of growth cones, 67.23 ± 2.67 %, collapsed if Nogo 20 was added to the neurons overexpressing the Pincher protein (Figure 4E). In contrast, growth cone collapse induced by Nogo 20 was fully abolished when PincherG68E was overexpressed. In this case, only 27.33 ± 1.79 of the growth cones showed a collapsed morphology (Figure 4E).

To exclude possible nonspecific effects of PincherG68E, we assessed the growth cone collapse response to a well known collapse inducing protein, Semaphorin 3A (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997; Luo et al., 1993; Tamagnone et al., 1999). More than 70% of the growth cones collapsed after the addition of Semaphorin 3A in Pincher as well as in PincherG68E overexpressing hippocampal neurons; there was no significant difference (75.48 ± 2.54 % and 70.06 ± 1.77 %, respectively; Figure 4F) between the two treatments.

These data strongly suggest that Pincher-dependent endocytosis is a crucial step in the chain of events that induce Nogo 20 specific growth cone collapse.

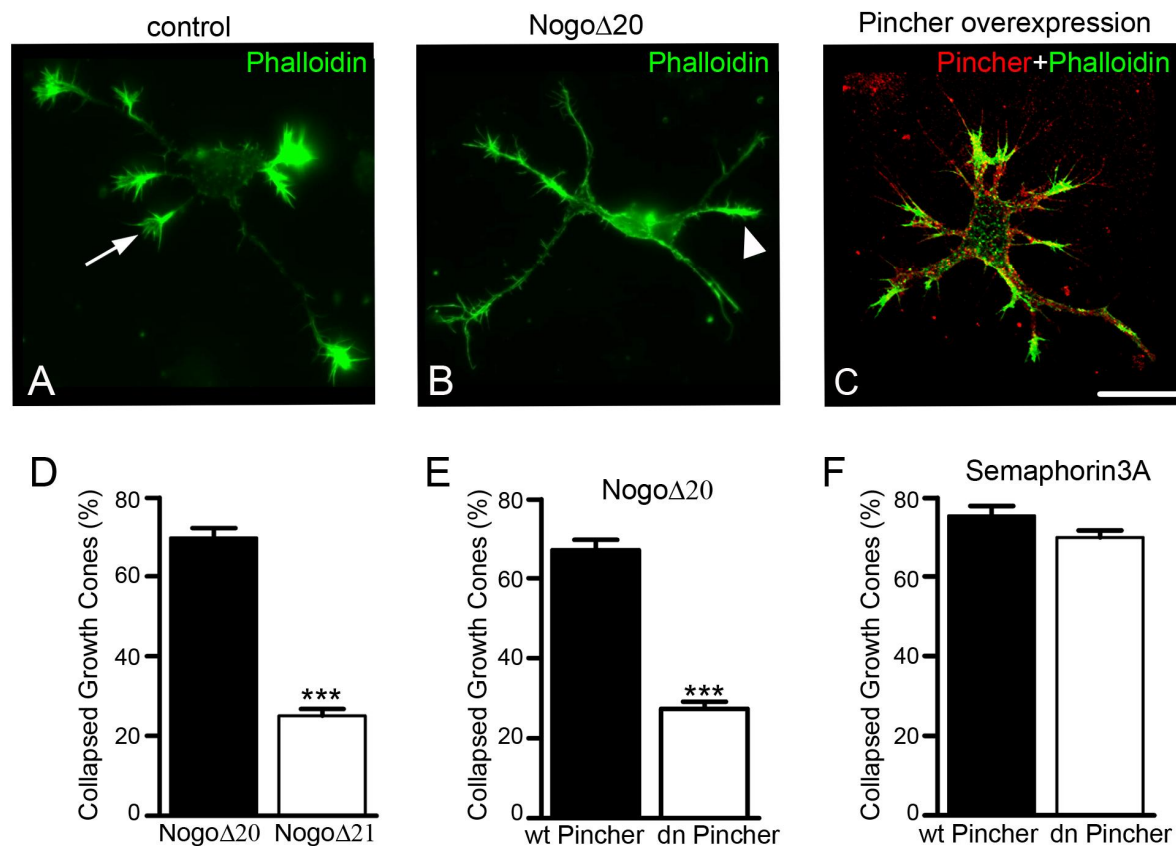


Figure 4. Endocytosis of Nogo 20 is required for Nogo 20 induced growth cone collapse in hippocampal neurons. Morphology of (A) non-collapsed (arrow) and (B) collapsed growth cones (arrow head) of E19 hippocampal neurons at DIV4 was visualized by staining of F-actin with Phalloidin-Alexa488 (green). (C) Hippocampal neurons infected with immunodeficient recombinant adenovirus containing HA tagged dn PincherG68E (red) were treated for 30 min with 300 nM Nogo 20; most growth cones remained uncollapsed (Phalloidin staining, Scale bars, 10 μ m). (D) Quantification of the proportion of collapsed growth cones after incubation with 300 nM Nogo 20 (black bars) or with 300 nM Nogo 21 (open bars). (E,F) Growth cone collapse of hippocampal neurons overexpressing either wt HA-Pincher protein (black bars) or mutant HA-PincherG68E protein (open bars) after incubation with 300 nM Nogo 20 (E) or 40 nM Semaphorin 3A for 30 min. Data represent the mean of three (Semaphorin 3A) or four (Nogo 20) independent experiments \pm SEM (number of neurons per group and experiment = 90). Asterisk marks highly significant difference between wt and dn Pincher infected hippocampal neurons (*** $P < 0.001$, Student's t test).

Inhibition of Pincher-dependent Nogo 20 internalization reduces Rho activation

The Rho family of small GTPases, which includes Rho, Rac and Cdc42, has important roles in regulating actin cytoskeletal dynamics. Nogo 20 activates RhoA, thus leading to disassembly of the actin cytoskeleton during growth cone collapse and growth inhibition (Niederost et al., 2002; Schwab, 2004). We investigated whether Rho activation could be dependent on Nogo 20 internalization. In order to measure activated Rho, we used GST-Rhotekin-RBD, which has been described to selectively bind to the active GTP-bound Rho proteins (Aspenstrom, 1999; Ren et al., 1999). Nogo 20 (300 nM) was added to PC12 cells for 30 min. Cells were then fixed, incubated with GST-Rhotekin-RBD, immunostained for GST and quantified by densitometry.

The comparison of the activated Rho levels of non-treated (Figure 5A) vs. Nogo 20 treated (Figure 5B) cells revealed a dramatic increase of Rho activation upon Nogo 20 addition (1.49 ± 0.03 vs. 1.00 ± 0.02 in control, $n = 149$ vs. 137 cells, $***P < 0.001$) (Figure 5C). However, when the macroendocytosis was blocked by the overexpression of dn PincherG68E, activation of Rho in PC12 cells remained at background levels after addition of Nogo 20 (1.12 ± 0.04 vs. 1.00 ± 0.02 in control, $n = 98$ vs. 137 cells) (Figure 5C). Reduced RhoA activation (23.12 ± 4.63 % of control, $n =$ three independent experiments) in cells overexpressing dn PincherG68E was confirmed in the Rho-A pull down assay (Figure 5D).

This finding that inhibition of Nogo 20 internalization interferes with Rho activation suggests that Nogo 20 containing vesicles may function as signalosomes, as described e.g. for NGF (Delcroix et al., 2003; Howe et al., 2001). To test this hypothesis, endosomal fractions were isolated from PC12 cells with and without prior addition of Nogo 20 and analyzed for their active RhoA content. Endosomal fractions from Nogo 20-treated cells exhibited higher RhoA activation compared to endosomal fractions from naive PC12 cells (Figure 7I). These data suggest that Nogo 20 may initiate the recruitment and assembly of specific signaling components including the small GTPase RhoA into so called "signalosomes".

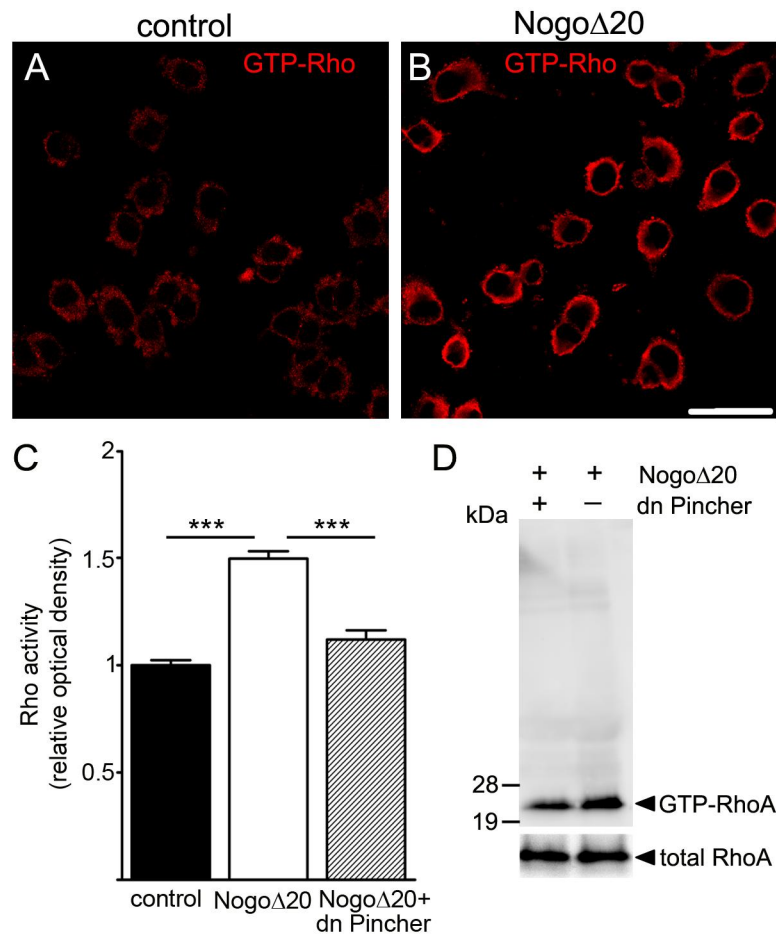


Figure 5. Nogo 20 induced Rho activation depends on internalization. Rho activation levels were examined in either (A) untreated (control) or (B) Nogo 20 (300 nM) treated PC12 cells for 30 min at 37°C. Active GTP-bound Rho was detected by incubation with GST tagged Rhotekin-RBD and immunostaining. Scale bar, 20 μ m. (C) Densitometric quantification of staining from three independent experiments. Data are normalized to the mean of the untreated group; asterisk marks highly significant differences between untreated, Nogo 20 treated or Nogo 20 and dn PincherG68E treated cells (three experiments; 30-50 cells per experiment; Student's *t* test, *** $P < 0.001$). (D) PC12 cells were either left untreated or transfected with dn PincherG68E construct. All cells were then incubated with 300 nM Nogo 20 for 30 min at 37°C. Extracted proteins were precipitated with Rhotekin-RBD beads. Precipitates were immunoblotted for RhoA (top panel). Total RhoA levels were determined from whole cell lysates as shown in the bottom panel.

Internalized Nogo 20 is retrogradely transported from neurites to cell bodies

To further test the role of Nogo- 20 endosomes, we examined whether Nogo 20 could be retrogradely transported from the axons to the cell bodies. Dissociated dorsal root ganglion (DRG) cells from E19 rats were cultured in compartmentalized chambers (Campenot, 1977) as indicated in Figure 6A. Within 7 days, fascicles of neurites grew under the Teflon ring and Silicon grease seals into the two side chambers and established a dense neuritic plexus. Nogo-20-T7 (300 nM) was then added to the side chambers. After 30 min or 6 hours, Nogo 20 was visualized by confocal microscopy. After 30 min of incubation, Nogo 20 fluorescence appeared in vesicular structures in the neurites of the distal compartments (Figure 6B, 6C) but was not detectable in proximal neurites and the cell bodies (Figure 6D, 6E). In contrast, after 6 hours of incubation, a large number of Nogo 20 containing vesicles appeared in proximal neurites and the cytoplasm of DRG cell bodies (Figure 6F, 6G), indicating the retrograde movement of endocytosed Nogo 20 from the neurites in the side chambers to the cell bodies in the centre chamber of the culture. Although majority of the cell bodies was positive for Nogo 20 (62.18 ± 6.74 %, $n =$ four independent experiments), some of the DRG cell bodies remained unlabeled (37.82 ± 3.36 %), showing that the fluorescent signals did not derive from leakage and direct uptake by cell bodies.

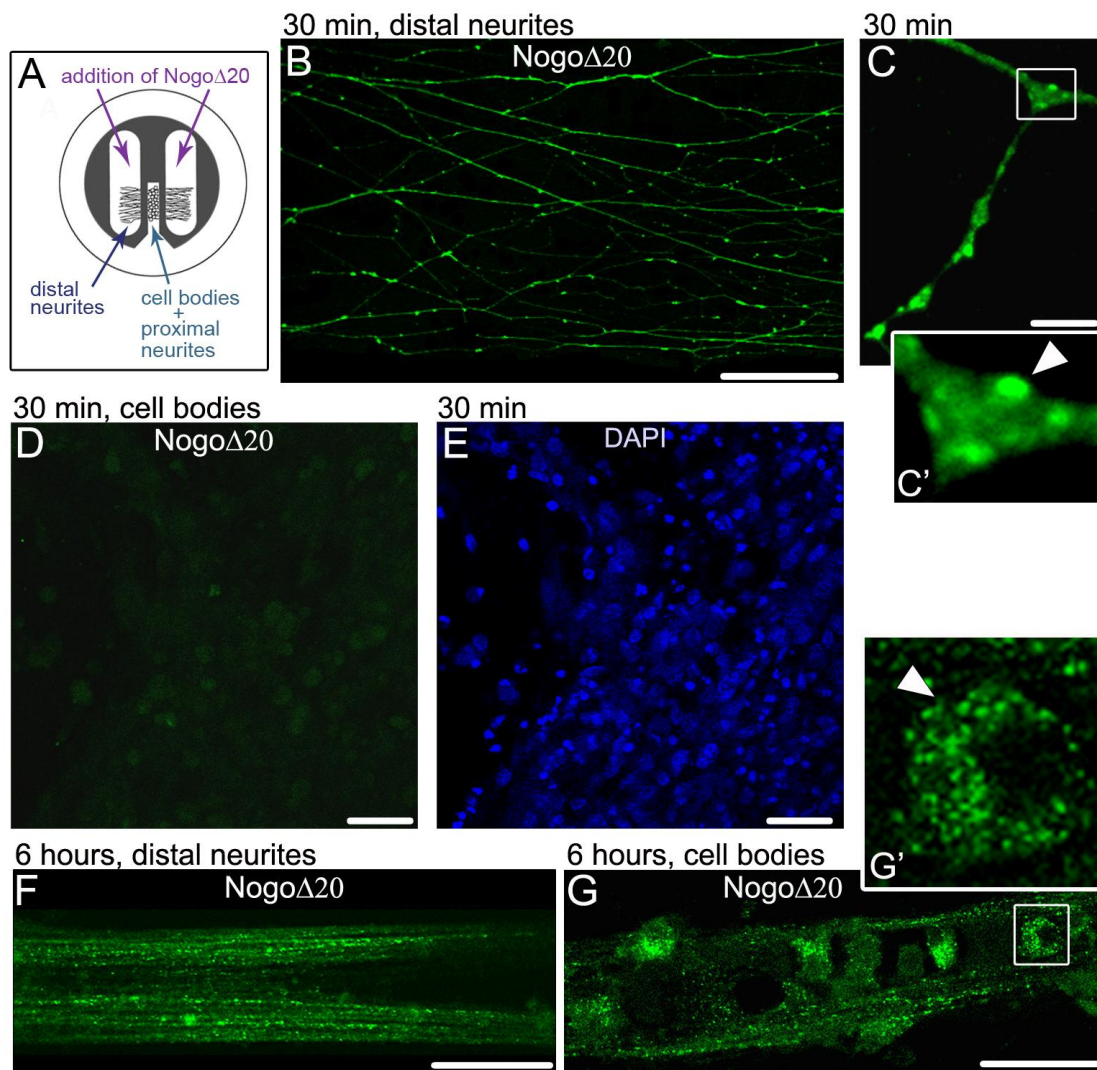
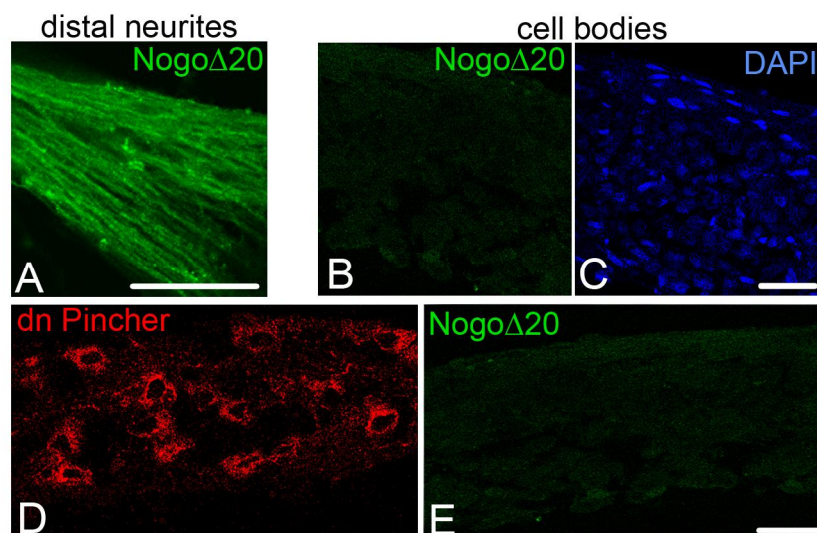


Figure 6. Upon internalization Nogo Δ 20 is retrogradely transported from the neurites to the cell bodies of dissociated DRG neurons. (A) Schematic representation of a compartmentalized chamber. (B) δ (G): Representative immunofluorescence images of retrogradely transported Nogo Δ 20 (300 nM) at indicated time points in dissociated DRG neurons which were cultured in compartmentalized Campenot chambers. (B) Uptake of Nogo Δ 20 (green) in the distal neurites at 30 min incubation. Scale bar, 20 μ m. (C) Nogo Δ 20 positive vesicles (green, arrow head) in the distal neurites. Scale bar, 10 μ m. (D) Nogo Δ 20 could not be observed in the cell body compartment 30 min after Nogo Δ 20 addition. (E) Cell bodies were stained with DAPI (blue). Scale bar, 40 μ m. Nogo Δ 20 positive distal neurites (F) and cell bodies (G) 6 hours upon addition of Nogo Δ 20 to the distal compartment. Arrowhead indicates Nogo Δ 20 positive vesicles in the cell body. Scale bars, 20 μ m.

Importantly, the blockade of retrograde transport with colchicine (Kreutzberg, 1969) completely abolished Nogo 20 detection in the DRG cell bodies, showing the involvement of active microtubule-dependent transport during Nogo 20 trafficking from the axons to the cell bodies (Supplementary Figure 2A- 2C).

As Pincher plays a crucial role in Nogo 20 endocytosis in PC12 cells, we analyzed the requirement of Pincher function on uptake and retrograde transport of Nogo 20 in DRG neurons. No Nogo 20 could be detected in the cell bodies of those DRG neurons overexpressing PincherG68E (Supplementary Figure 2D, 2E). The overexpression of PincherG68E completely abolished the retrograde trafficking of Nogo 20, confirming that the Pincher protein is required for neuronal Nogo-A uptake and trafficking.



Supplementary Figure 2. **Retrograde transport of Nogo 20 is microtubule and Pincher dependent**

(A-C): Retrograde transport of Nogo 20 in presence of colchicine. Cell bodies of DRG neurons cultured in Campenot chambers were pretreated with colchicine for one hour prior to the addition of Nogo 20-T7. 6 hours after Nogo 20 addition, Nogo 20 (green) was found in the distal compartment (A) but was absent from the cell body compartment (B). Cell bodies were detected with DAPI (C, blue) Scale bars, 40 μ m. (D) The cell body compartment of dissociated DRG neurons was infected with immunodeficient adenovirus containing the dn HA-PincherG68E at DIV5. At DIV7 Nogo 20 was added to the distal neurite compartment and after 6 hours neurons were immunostained for Pincher (red) and Nogo 20 (green). No retrogradely transported Nogo 20 could be observed in the cell body compartment. Scale bar, 20 μ m.

Retrogradely transported Nogo 20 activates RhoA en route and decreases pCREB levels in the DRG cell bodies

Next, we asked if Rho activation can be observed in DRG neurites and cell bodies after internalization of Nogo 20. Active Rho was measured with GST-Rhotekin-RBD protein as described earlier in the distal neurites or cell bodies of the compartmentalized DRG cultures, 30 min and 6 hours after addition of 300 nM Nogo 20 to the distal neurite compartments. We found Rho activation in the neurite compartments 30 min after Nogo 20 addition. Interestingly, active Rho was precisely co-localized with Nogo 20 positive vesicles in the neurites (Figure 7A-C; 86.58 ± 14.48 % of overlap, $n = 26$ neurites). In the cell body compartment, the Rho activation was not seen at 30 min (Figure 7D, 7E) but was prominent at 6 hours after Nogo 20 addition (Figure 7G). Thus, the temporal activation of Rho-A reflects the retrograde transport of Nogo 20 suggesting the formation of Nogo 20/RhoA signalosomes.

The activation of the cAMP response element binding protein CREB, e.g. by neurotrophins, can overcome inhibitory effects of myelin-associated neurite outgrowth inhibitors (Gao et al., 2004). To test whether Nogo 20 influences CREB signaling, we determined the levels of phosphorylated CREB (pCREB) upon Nogo 20 addition. The low basal level of pCREB in DRG cell bodies makes these neurons highly responsive to increases in pCREB, which occurs after application of neurotrophins to the axons (Watson et al., 1999). Since our DRG neurons are cultured in compartmentalized chambers in presence of NGF, we detected high basal levels of pCREB (Figure J). Addition of Nogo 20 to the distal compartments for 6 hours resulted in a dramatic decrease (22.39 ± 4.47 % of control, $n =$ three experiments) of pCREB in the cell body compartment (Figure 7J).

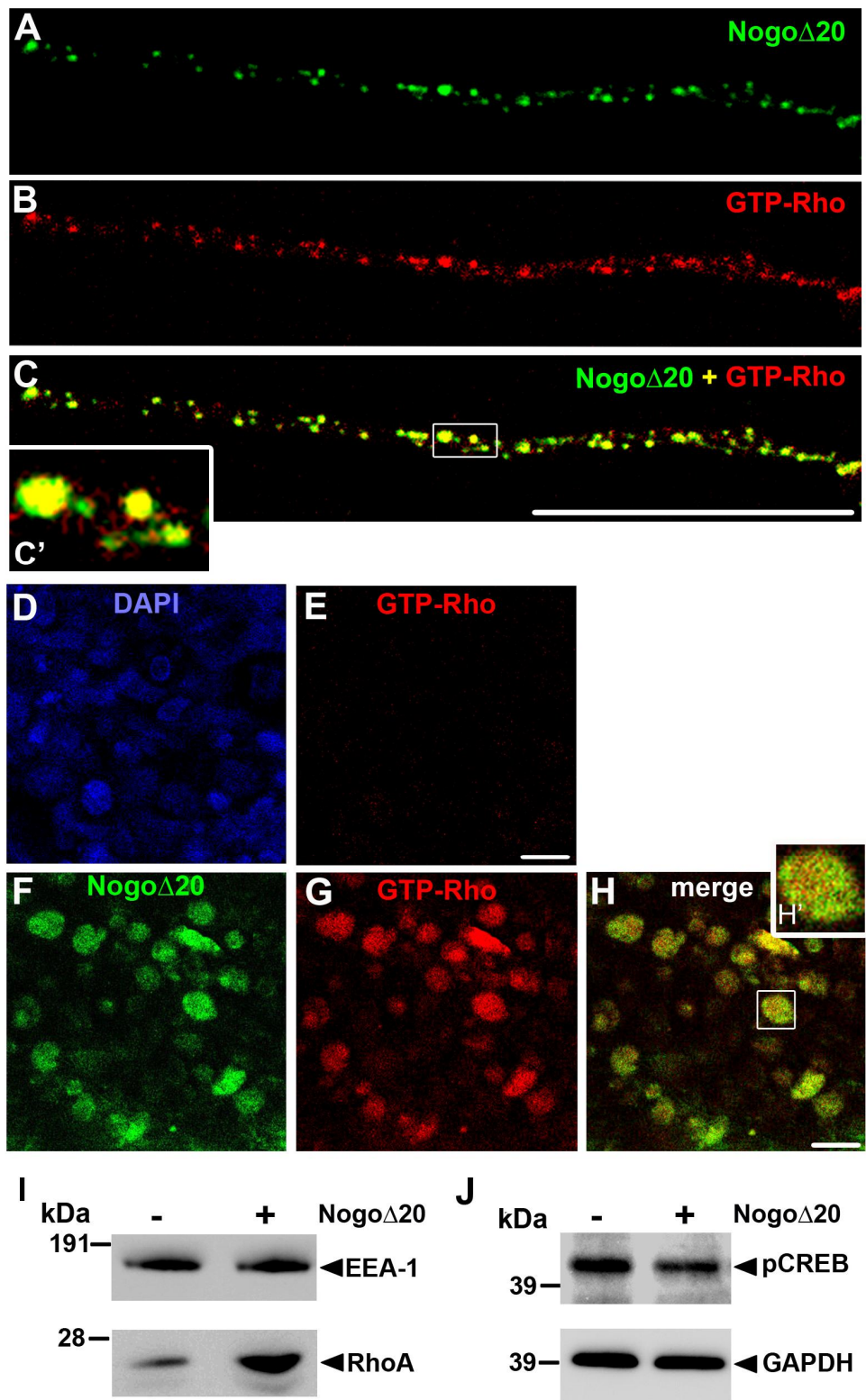


Figure 7. Nogo 20 triggers Rho activation en route to DRG cell bodies

Active GTP bound Rho was visualized in the distal neurites and the cell body compartment of Campenot chambers upon addition of Nogo 20 (300 nM) to the distal compartment for either 30 min or 6 hours. (A)-(C) 30 min after Nogo 20 addition activated Rho (red) co-localizes with Nogo 20 positive vesicles in the neurites of the distal compartment of the Campenot chambers. Cell bodies stained with (D) DAPI (Blue) were negative for activated Rho (E; red). (F)-(H) 6 hours upon Nogo 20 (green) addition activated Rho (red) was also observed in the cell body compartment. Scale bars, 10 μ m. (I) Early endosome containing F1 sucrose density fractions of untreated (control) or Nogo 20 treated (30 min at 37°C) PC12 cells were reacted with Rhotekin-RBD beads. The bound proteins were immunoblotted with an anti-Rho A monoclonal antibody (bottom panel). (J) Cell bodies from DRG neurons cultured in Chambers were collected 6 hours after Nogo 20 addition and immunoblotted for pCREB. Addition of Nogo 20 decreased the pCREB levels.

Discussion

Many studies on endocytosis and retrograde transport of ligand-receptor complexes in neurons have focused the factors promoting survival and plasticity, in particular the neurotrophins (Campenot and MacInnis, 2004; Ginty and Segal, 2002; Howe and Mobley, 2005). However, during development, in the adult nervous system and after injury axons are also exposed to growth inhibitory signals, a prominent one being Nogo-A. Up to now, nothing was known about whether the myelin-associated neurite growth inhibitor Nogo-A can signal through endosomes, which may also be retrogradely transported in neurons. We used Nogo 20, the most potent fragment of Nogo-A that mediates growth cone collapse through a receptor other than NgR, to examine the role and mechanism of endocytosis. We find that Nogo 20 is endocytosed in a non-conventional manner, using Pincher- and Rac-mediated macroendocytosis. Our study indicates that endocytosis of Nogo 20 is essential to mediate growth cone collapse. The inhibition of Pincher-mediated Nogo 20 endocytosis prevented Nogo 20 induced, but not Semaphorin 3A induced growth cone collapse and diminished Nogo 20-triggered RhoA activation. Furthermore, Nogo 20 is taken up by DRG neurites and retrogradely transported to the cell bodies where it activated Rho and reduced pCREB levels. Nogo 20 positive endosomal vesicles were positive for activated Rho in the neurites, suggesting that Nogo 20 signalosomes transmit growth-inhibitory signals to the soma.

Most cell surface proteins and receptor-bound ligands are internalized through the clathrin coated vesicle or the caveolin pathways (Le Roy and Wrana, 2005; Mayor and Pagano, 2007). We found that Nogo 20 internalization does not depend on the Nogo66 receptor NgR as shown by pharmacological blockage of NgR with PI-PLC or NEP1-40. Surprisingly, by overexpression of the dominant negative constructs eps15 E 95/295 and dynamin K44A which interfere with the clathrin and caveolin endocytosis machinery, we found that neither of the two pathways mediates Nogo 20 endocytosis. In contrast, overexpression of a mutant form of the Pincher protein in which the P-loop ATP binding site is destroyed by mutation G68E (Shao et al., 2002) almost completely blocked the internalization of Nogo 20, indicating a requirement of Pincher ATPase activity for this process. Pincher protein has been implicated in the clathrin-independent, Rac

mediated and pinocytosis-like uptake of the activated neurotrophin receptor complex (Shao et al., 2002). The requirement of Pincher protein and the small GTPase Rac for Nogo 20 internalization leads to conclusion that Nogo 20 is similarly macroendocytosed. Given that Pincher has recently been classified as a member of the dynamin superfamily, in this case it may replace dynamin in mediating macroendocytosis (Daumke et al., 2007). Macroendocytic signaling may have more general implications in growth inhibition, because EphrinB-EphB containing vesicles, which do not co-localize with the known markers of the clathrin and caveolin endocytic pathways (Marston et al., 2003), may also be internalized via Rac-dependent macroendocytosis (Marston et al., 2003).

Using growth cone collapse as a functional readout, we addressed the question whether Nogo 20 internalization is necessary for Nogo 20 signaling. Blockade of Nogo 20 endocytosis by dominant negative PincherG68E prevented Nogo 20 induced growth cone collapse of hippocampal neurons. Under the same conditions Semaphorin 3A induced growth cone collapse was not affected. Importantly, the Nogo 20 induced growth cone collapse is known to occur with a slower time course than the collapse elicited by Semaphorin 3A (Oertle et al., 2003), suggesting different signaling cascades evoked by the two proteins. For Nogo-A, the first morphological changes can be observed after 90 s and the collapse is complete after 20-40 min (Bandtlow et al., 1993; Oertle et al., 2003). Interestingly, this time course matches that of Nogo 20 endocytosis: first Nogo 20 containing vesicles appeared after 2 min of exposure to Nogo 20 (data not shown), and a large number of Nogo 20 endosomes with early endosome marker accumulated intracellularly after 30 min. This slow time course of endocytic processing is similar to that found for neurotrophin, as opposed to epidermal growth factor receptor (EGFR) endocytic signaling, and may be a general feature of Pincher-mediated endocytic signaling (Valdez et al., 2007). Importantly, members of another repulsive protein family, the Ephrins were also shown to depend on endocytosis for growth cone collapse: In the absence of Vav proteins, ephrin-Eph endocytosis was blocked, resulting in defects in growth collapse *in vitro* and significant defects in retinogeniculate axon guidance *in vivo* (Cowan et al., 2005).

Our finding that prevention of Nogo 20 internalization also blocked the activation of Rho proteins in PC12 cells and DRG neurons provides a second line of evidence that Nogo 20 internalization is required for Nogo 20 signal propagation. Accordingly, subcellular fractionation revealed that Nogo 20 containing early endosomes contain activated RhoA-GTPase, indicating that Nogo 20 signals after internalization. Consistent with this, we find that Nogo 20 containing vesicles were transported retrogradely from the neurites to the cell bodies of DRG neurons cultured in compartmentalized chambers. Activated Rho co-localized with Nogo 20 positive vesicles in these DRG neurites. As high Rho activity in the DRG cell bodies was not observed after 30 min but prominently after 6 hours of Nogo 20 addition, we conclude that Nogo 20 vesicles activate Rho also en route in the cell bodies. Taken together, these data strongly indicate the formation of Nogo 20 signaling endosomes which are retrogradely transported along the axons.

Nogo-A is predominantly found in the innermost, adaxonal membranes around axons in intact CNS (Huber et al., 2002; Wang et al., 2002b). One question which arises from this study is whether myelin-associated full length Nogo-A is transcytosed from the myelin into the axons or whether Nogo-A undergoes protease-dependent cleavage to release Nogo 20 fragments which are taken up by surrounding axons. The existence of soluble Nogo-A fragments in cerebrospinal fluid from patients with multiple sclerosis (Jurewicz et al., 2007) and the cleavage of Nogo-A upon optic nerve injury (Ahmed et al., 2006) speak in favor of the second mechanism. However, we can not exclude that full-length transmembrane Nogo-A may be transcytosed as it has been described for EphB-ephrinB complexes (Zimmer et al., 2003).

Given our finding that Nogo-A signals retrogradely and the constant contact of Nogo-A with the axon, it is tempting to speculate that in the intact CNS Nogo-A signals originating along the axons tonically suppress axonal growth. Once the axon has reached its target and myelination starts, Nogo-A starts retrogradely communicate to the cell body that the growth machinery is not needed any longer. Transitory growth responses of Purkinje axons and the corticospinal tract in intact rats upon Nogo-A neutralization support this concept (Bareyre et al., 2002; Buffo et al., 2000; Gianola et al., 2003).

In many respects Nogo-A seems to counteract the effect of neurotrophic factor signaling. While e.g. NGF promotes neurite outgrowth and attracts growth cones, Nogo-A inhibits outgrowth and induces growth cone collapse. Retrograde trafficking of NGF signaling endosomes is associated with an increase in pCREB in cell bodies (Cox et al., 2008; Riccio et al., 1997). In contrast, we find that retrograde trafficking of Nogo 20 is associated with a decrease of pCREB levels. It seems that both proteins use a similar endocytic machinery to achieve opposing cellular responses, suggesting that different endosome-based signals are integrated in the soma after retrograde transport. In the future, characterization of the ligand-receptor complexes and of retrogradely activated genes and more precise analysis of local and distal effects induced by Nogo-A in neurons may help us understanding the dualistic roles of NGF and Nogo-A.

Materials and Methods

Cell Culture

PC12 cells were grown in DMEM media (Gibco) supplemented with 6% new born calf serum and 6% horse serum. Cells were transfected with Lipofectamin2000 (Invitrogen) in OptiMEM (Gibco).

Primary hippocampal neurons derived from rat embryos were cultured as described previously (Kaeck and Banker, 2006). In brief, the hippocampi of E19 rats were dissected, digested (0.05% papain; Sigma) and washed in PBS. Cells were then dissociated and 6,000-8,000 cells were plated onto poly-L-lysine and laminin coated 18-mm glass coverslips in 12 well cell culture plates containing glial feeder layer in neurobasal/B27 medium (Gibco/Invitrogen).

Dissociated DRG neurons derived from E19 rats were cultured in Campenot Chambers as described previously (Campenot, 1977). Briefly, E19 dissociated DRG neurons were plated in the cell body compartment in neurobasal/B27 medium supplemented with 100 ng/ml NGF and 250 μ M cytosine arabinoside (1- β -D-arabinofuranosylcytosine) to inhibit glial growth. At DIV4 media was changed and compartments were checked for bulk leakage. Cultures from leaking chambers were excluded from further study. Retrograde transport experiments were performed at DIV7, when neurites had crossed the divide into the distal neurite compartment.

DNA and viral constructs

Dynamin II K44A-GFP construct was obtained from M. McNiven (Mayo Clinic, Rochester, USA), Eps15 (95-295)-GFP construct was obtained from A. Benmerah and A. Dautry-Varsat (Institut Pasteur, Paris, France) and Rac1-T7 (wt and N17 mutant) constructs were obtained from D. Bar-Sagi, (New York University, New York, USA).

Defective adenoviruses containing Pincher-HA (Shao et al., 2002) constructs were used. For efficient infection 50 MOI (multiple of infection) were used. Hippocampal neurons were infected at DIV2 and analyzed at DIV4. The cell body compartments of dissociated DRG neurons were infected at DIV5 and the cultures were grown for further 2 DIV to achieve high level of protein overexpression.

Drug treatments

Cells were preincubated for 60 min at 37°C in serum-free DMEM containing 1U/ml PI-PLC (Invitrogen) or 1 μ M NEP1-40 (Alpha Diagnostics). Preincubation with 25 μ g/ml nystatin (Sigma-Aldrich) plus 10 μ g/ml progesterone (Sigma-Aldrich) was performed over night. The drugs were present throughout the experiments. Cell bodies of DRG neurons were preincubated with 125 nM colchicine (Sigma-Aldrich) 1 hour prior to addition of Nogo 20.

Growth cone collapse

The response of neuronal growth cones was quantified on three independent experiments (n=90 neurons per group from each experiment). At DIV4 hippocampal neurons were incubated with either 300 nM Nogo 21 (control) or 300 nM Nogo 20 or 40 nM Semaphorin 3A for 30 min at 37°C, fixed and stained for F-actin with Phalloidin-Alexa 488 to visualize the growth cone morphology. The collapsed growth cones of each neuron, which were defined as those with no lamellipodia and not more than two filopodia (Kapfhammer et al., 2007), were counted and expressed as percentage of the total growth cones of the belonging neuron. The infection with defective adenoviruses containing HA-Pincher constructs was performed at DIV2 with 50 MOI. After 1 h medium containing adenoviruses was removed and the cells were cultured for another 2DIV prior to collapse experiment. Only HA-Pincher positive neurons which were detected with anti-HA immunostaining were analyzed in the growth cone collapse experiment.

Internalization assay and immunofluorescent microscopy

For internalization assays PC12 cells were serum starved 3 hours prior to addition of proteins for 30 min at 37°C. Proteins were used at following concentrations: Nogo 20 and Nogo 21 at 300 nM, transferrin-biotin (Invitrogen) at 1 µg/ml and cholera toxin B-A594 (Invitrogen) at 1 ng/ml. Cells were then fixed with 4% paraformaldehyde for 15 min and subsequently permeabilized with 0.1% Triton X-100 for 30 min at RT. After blocking cells were incubated first with primary antibodies for 30 min, washed 3 times for 5 min and then incubated with secondary antibodies for 30 min.

Coverslips were mounted with Dako (Dako Cytochrom). Images were acquired on a Leica type DM RE microscope using a confocal scanning system SP2 or SP5 from Leica equipped with 40x/1.25, 63x/1.4 and 100x Planchromat objectives. The thickness of all confocal slices varied between 0.2 and 0.8 µm.

Images were processed with the use of Photoshop (Adobe Inc., Mountain View, CA). Colocalizations were analyzed with the colocalization module of Imaris (Bitplane, Zurich, Switzerland). Data were given as the mean value \pm SEM. Data analysis was performed by Prism 4.0 (GraphPad software) using independent Student's *t* test.

The following primary antibodies were used: rabbit anti-EEA-1 (Abcam) 1:250, mouse anti-T7 (Novagen) 1:1000, rabbit anti-Pincher (Shao et al., 2002) 1:1000, rat anti-HA (Roche) 1:250. Secondary antibodies: Alexa-488, Alexa-594, Avidin-Rhodamin and Avidin-FITC were from Molecular Probes. All immunostaining experiments were repeated at least three times.

Recombinant fusion proteins

Recombinant fusion proteins Nogo 20-T7 and Nogo 21-T7 were purified as described previously (Oertle et al., 2003). In brief, *Escherichia coli* BL21/DE3 were transformed with the bacterial expression vectors pET28 and grown in 2x YT medium (Invitrogen). Expression of the fusion proteins was induced by addition of 1 mM isopropyl-1-thio- β -D-galactopyranoside (IPTG) to log phase culture at 30°C for 4 hours. The highly expressed fusion proteins were purified using the Co²⁺-Talon Metal Affinity Resin (Clontech). Upon elution with 250 mM imidazole proteins were extensively dialyzed against PBS, pH 7.4. The purity of the recombinant proteins was confirmed by SDS-PAGE and Coomassie Brilliant Blue® staining. The protein concentration was determined with BCA protein assay kit (Pierce) using bovine serum albumin as a standard. In addition, Fc-tagged Nogo 20 fragment was used (R&D systems).

RhoA pull down assay and immunostaining

PC12 cells were homogenized in RIPA buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl₂, protease inhibitor cocktail (Complete Mini; Roche Diagnostics), 1 mM PMSF). After centrifugation for 20 min at 13 000g at 4°C homogenates (0.5 mg/ml) were incubated for 1 hour at 4°C with 60 μ g GST-Rhotekin RBD beads (Cytoskeleton, Inc.). The beads were then washed twice and eluted in sample buffer. GTP-bound RhoA and total RhoA present in the cell lysates were immunoblotted with mouse anti-Rho-A antibody (Santa Cruz Biotechnology, Inc) 1:200.

Activated Rho was detected by probing cells with the Rho-binding domain from the Rho-GTP-interacting protein rhotekin-RBD tagged with GST (Cytoskeleton, Inc.) 20 μ g/ml for 1 hour at 37°C. Rabbit anti GST (Abcam) 1:400 was subsequently used.

Subcellular fractionation

PC12 cells were cultured on 15 cm plates, serum starved (DMEM/BSA) for 3 h and either left untreated or treated with Nogo 20-T7 for 30 min. Next, cells were homogenized in a detergent-free manner with a 22-G needle in the homogenization buffer (250 mM sucrose, 3 mM imidazole, 1 mM EDTA, protease inhibitor cocktail (Complete Mini; Roche Diagnostics), and 0.03 mM cycloheximide, pH7.4). Subsequently, a post-nuclear supernatant (PNS) was prepared according to standard techniques (Bomsel et al., 1990). The PNS was adjusted to 40.6 % sucrose, loaded at the bottom of an SW60 centrifuge tube (Beckman), and overlaid sequentially with 1.5 volumes of 35% and 1 volume of 25% sucrose solutions in 3 mM imidazole, 1mM EDTA, pH 7.4. The rest of the tube was filled up with homogenization buffer. The gradient was then centrifuged at 210 000g at 4°C for 90 min using an SW60 rotor (Beckman). After centrifugation, different interfaces and sucrose cushions were collected from top to bottom of the tube. The protein concentration of each fraction was determined with BCA protein assay kit (Pierce) using bovine serum albumin as a standard. Equal amounts of protein from each fraction were loaded on SDS-PAGE, followed by immunoblotting with mouse anti-EEA1 (BD Transduction Labs) 1:500, mouse anti-nucleoporin p62 (BD Transduction Labs) 1:1000, mouse anti-T7 (Novagen) 1:5000 and mouse anti-RhoA (Santa Cruz Biotechnology, Inc.) 1:200. This experiment was repeated three times with similar results.

WesternBlot Analysis

Proteins (25-100 µg) were separated by electrophoresis on a 4-12% polyacrylamide gel and transferred to nitrocellulose membranes. Blots were preincubated in a blocking solution of 3% Top Block (VWR International) in TBST (0.1 M Tris base, 0.2% Tween 20, pH 7.4) for 1 h at room temperature, incubated with primary antibodies overnight at 4°C and after washing, with a horseradish peroxidase-conjugated anti-rabbit antibody (Pierce Biotechnology) 1:10 000. CREB was detected with rabbit anti pCREB (Millipore Bioscience Research) 1:500 and anti-GAPDH (Abcam) 1:10 000. Protein bands were detected by adding SuperSignal West Pico Chemiluminescent Substrate (Pierce) by exposing the blot in a Stella detector (Raytest).

Densitometry analysis was performed with NIH software and by normalizing the band intensities to GAPDH values.

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“The important thing is not to stop questioning. Curiosity has its own reason for existing.”

Albert Einstein

Chapter 3

Neurotrophins and Nogo-A: Antagonistic regulators of neurite outgrowth?

3.1 Overview

The study presented in this thesis explored the significance of endocytosis for the function of Nogo-A. The results shown in Chapter 2 clearly illustrate the requirement of Nogo 20 internalization for Nogo-A signaling. Inhibition of Nogo 20 internalization prevented growth cone collapse and Rho-A activation. These experiments also demonstrated that the internalization of Nogo 20 does not follow the conventional clathrin- and dynamin-dependent pathway, but rather depends on the Pincher protein. In addition, these data strengthen the previous findings that Nogo 20 acts by a specific mechanism which is independent of NgR. Further, these data provide the first evidence that Nogo 20 induces the formation of signalosomes containing Rho-A, which are retrogradely transported from the axons to the cell bodies where they decrease pCREB levels. The intriguing finding that Nogo 20 acts on pCREB in the opposite manner to NGF suggests a potential mechanism for Nogo 20 and NGF to act antagonistically. A schematic representation of the Nogo-A signaling endosome hypothesis is shown in Figure 1.

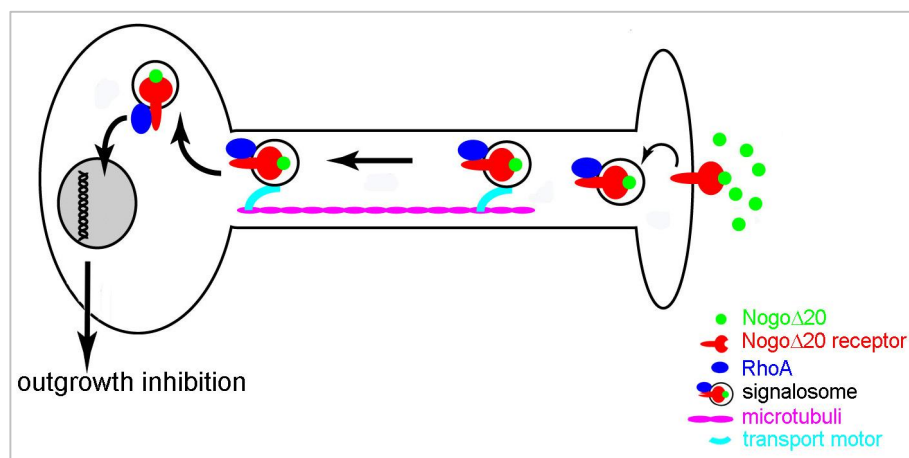


Figure 1: Schematic representation of Nogo-A signalosome pathway
(adapted from Howe, 2005)

Upon binding to its receptor, Nogo 20 induces the formation of signaling endosomes which contain activated RhoA. The signalosomes are then transported via a microtubule dependent mechanism from the axons to the cell bodies where they initiate transcriptional changes leading to neurite outgrowth inhibition.

3.2 Implications and future directions

The role of endocytosis in the nervous system has been demonstrated so far for neurotrophic factors and ephrins (Cowan et al., 2005; Howe and Mobley, 2005; Marston et al., 2003; Zimmer et al., 2003). Internalization of myelin-associated neurite outgrowth inhibitors has not yet been shown. This study reports that the potent, myelin derived growth inhibitor Nogo-A can induce the formation of endosomes and retrograde signaling in neurons. The retrograde transport of the active, inhibitory Nogo 20 fragments raises interesting questions: (i) what are the transcriptional cell body changes upon retrograde transport of Nogo 20?, (ii) does the Nogo 20 signalosome contain other downstream components besides RhoA?, (iii) does a soluble, active Nogo 20-fragment exist *in vivo*?, and (iv) under which biological conditions does retrograde signaling take place?

3.2.1 Identification of Nogo 20 mediated transcriptional changes

The retrograde transport of NGF signalosome leads to transcriptional changes in the cell body of neurons. Among the regulated transcription factors, CREB and SRF are best characterized (Cosker et al., 2008). The activation of CREB dependent genes results in a survival response (Riccio et al., 1997) whereas the activation of SRF dependent genes results in an axonal growth response (Wickramasinghe et al., 2008). The transcriptional changes evoked by Nogo-A are not yet known. A comprehensive gene chip analysis of the cell bodies at different time points upon addition of Nogo 20 would lead to identification of potential gene candidates which might be involved in the outgrowth inhibition response.

The opposed effects on pCREB levels induced by Nogo-A in comparison to NGF in DRG neurons suggest an antagonistic growth response of the two proteins which might be reflected in the opposing regulation of some genes. An alternative to the gene chip analysis would be the examination of gene candidates which are shown to be regulated by NGF signaling. One promising candidate would be the serum response factor, SRF since it mediates NGF-dependent

axonal growth, branching, and target innervation by regulating gene expression of cytoskeletal genes such as *actin* and *actin* but also LIM family regulators such as *Ldb2* (Wickramasinghe et al., 2008). Genetic ablation of Nogo-A results in enhanced expression of cytoskeleton related proteins such as actin (, ,), GFAP or neurofilament-68, and in enhanced growth cone motility by regulation of the LIM kinase (LIMK) pathway through cofilin (Montani et al., 2009). It is tempting to speculate that Nogo-A and NGF signaling pathways converge at the level of SRF and thus on the regulation of the LIMK pathway. To verify the involvement of CREB and SRF in the neurite outgrowth, it would be important to test whether the activation of these factors can directly overcome the inhibitory role of Nogo 20.

3.2.2 Characterization of Nogo 20 signalosome

In addition to the gene chip approach, a complementary proteomic analysis of the Nogo 20 signalosome could shed more light on the downstream signaling of the Nogo-A specific receptor. In addition to ligand and receptor, growth factor signalosomes contain several downstream signaling components (Ginty and Segal, 2002). The isolation of Nogo-A specific signalosomes may lead to identification of new signaling components and thus contribute to a better understanding of the inhibitory signals.

3.2.3 Nogo-A fragmentation

Although Nogo 20 is a highly inhibitory Nogo-A fragment, its existence *in vivo* has not been shown. The existence of soluble Nogo-A fragments in cerebrospinal fluid from patients with multiple sclerosis (Jurewicz et al., 2007) and the cleavage of Nogo-A upon optic nerve injury (Ahmed et al., 2006) suggest a possible protease-dependent cleavage of Nogo-A. Since the BACE-1 co-localizes with some members of the reticulon family i.e. RTN3 and RTN4B/Nogo-B, it could be possible that it can also cleave Nogo-A. It would be interesting to test whether the

inhibition of BACE might interfere with neurite outgrowth inhibition or growth cone collapse mediated by Nogo-A.

However, we can not exclude that full-length transmembrane Nogo-A may be transcytosed in the form of a pinched-off vesicle from the donor to the responsive cell, as it has been described for EphB-ephrinB complexes (Zimmer et al., 2003).

3.2.4 Possible role of Nogo-20 signalosomes in the adult normal CNS

Given the Nogo-A localization in the innermost, adaxonal myelin membranes around axons in the intact CNS (Huber et al., 2002; Wang et al., 2002b) and their constant contact with the axons, it is tempting to speculate that in the intact CNS Nogo-A signals originating along the axons tonically suppress axonal growth. In agreement with this hypothesis, it has been shown that Nogo-A neutralization results in a transitory growth responses e.g. of Purkinje axons and the corticospinal tract (Bareyre et al., 2002; Buffo et al., 2000; Gianola et al., 2003). The enhanced growth response in mature intact hippocampal slice cultures upon acute Nogo-A neutralization supports this idea (Craveiro et al., 2008). In addition, the plasticity of the visual cortex is extended beyond the normal postnatal critical period in mice lacking NgR or Nogo-A/B (McGee et al., 2005). According to these findings one could imagine the following scenario: once the axon has reached its target and myelination starts, Nogo-A begins to retrogradely communicate to the cell body that the growth machinery is not needed any longer. Whether Nogo-20 signalosomes exist in the intact CNS axons remains to be shown.

3.2.5 Retrograde axonal transport in the injured CNS

Axonal injury evokes a cell body response which initially includes increase in gene transcription and protein translation (Ambron and Walters, 1996). An elevated expression of the growth associated protein 43 (GAP 43) mRNA has been observed in axotomized neurons. However,

after three week these mRNA levels decline (Verhaagen et al., 1993). One possible explanation for this transitory growth boost would be the lack of retrograde transported Nogo 20. Upon injury, the contact of Nogo-A with the axons is disrupted and thus the growth inhibitory Nogo 20 signal abolished. In turn the growth machinery is switched on and the axotomized neurite is looking for new connection. Once the seeking axon encounters myelin, the retrograde transport of Nogo-A endosomes would start again to suppress the neurite growth machinery. To better understand these injury signals, the role of the Pincher protein could be addressed. It would be interesting to know if the inhibition of the retrograde transport of Nogo-A by down-regulation of the Pincher protein would lead to a better recovery upon spinal cord injury. However, an open question remains as to whether full-length Nogo-A or only the Nogo 20 fragments are transported *in vivo*. Some experimental analysis may help to shed more light on this issue. If Nogo-A would be cleaved, it should be possible to detect the Nogo-A fragmentation by isolating it from the injured nerves.

3.2.6 Possible antagonistic effects of Nogo-A and neurotrophins during development

Neurotrophins have been implicated in axon guidance and target innervation during neuronal development (Huang and Reichardt, 2001). The local expression of target-derived neurotrophic factors such as NGF, BDNF and NT-3 in target tissue can influence the extent of terminal arborization within the target region during development (Markus et al., 2002). Transgenic mice over-expression of BDNF in epithelial tissue show an increase in peripheral innervation densities (Albers et al., 1996; Davis et al., 1994). Selective deletion of BDNF receptor TrkB in the pyramidal neurons leads to altered dendritic arborization in cortical layers (Xu et al., 2000). Since local application of Nogo-A leads to growth cone collapse and the zebrafish embryos lacking Nogo orthologues show severe defects in axon pathfinding, it is tempting to speculate that Nogo-A might play a restrictive role in target innervation. One expectation would be that the down-regulation of Nogo-A would result in higher target innervation densities. The mice lacking Nogo-A do not show any serious behavioral defects (Willi et al., 2009). However, absence of Nogo-A induces increased axonal branching of cortical neurons *in vitro* (Mingorance-Le Meur et

al., 2007). It is very possible that the genetic ablation of Nogo-A results in the up-regulation of other proteins which compensate for the role of Nogo-A. To overcome these compensatory effects, it is important to examine the role of Nogo-A in the target innervation by acute neutralization of Nogo-A *in vivo*. The chicken embryo is an attractive experimental system because of its accessibility during different developmental stages and the possibility of applying functional RNA interference by *in ovo* electroporation or of injection of Nogo-A neutralizing antibodies. Since Nogo-A is expressed on muscle cells during early development, one would down- or up-regulate Nogo-A in the muscle cells and analyze the muscle innervation. An alternative experimental system for the CNS is the visual system. The retinotopic projections of ganglion cells from distinct positions in the retina to the superior colliculus have been extensively studied with ephrins, a family of repulsive guidance molecules (Luo and O'Leary, 2005). At E 15, during retinocollicular development when the retinal ganglion cell axons arrive at the chiasm, Nogo-A has been shown to be most prominent on radial glia at the optic nerve head and the chiasmatic midline. NgR has been localized on the axons of the optic tract (Wang et al., 2008). These expression patterns suggest an involvement of Nogo-A in the routing of axons in the optic chiasm. For example, Nogo-A might selectively inhibit the NgR expressing retinal axons to cross the midline. Since the mouse embryo is not easily accessible, an inducible gene knock out would be essential.

Another possible role for Nogo-A during development is axon pruning. During nervous system development, over-production of neurons and axons is followed by a regressive phase in which the inappropriate axonal branches are eliminated (Luo and O'Leary, 2005; Raff et al., 2002). Axonal pruning has been studied *in vitro* by using compartmentalized chambers (Campenot, 1977). NGF depletion from the axonal compartment results in a rapid axonal fragmentation (MacInnis and Campenot, 2005). If Nogo-A would oppose the effects of NGF, one would expect Nogo 20 to mimic the effects observed upon NGF deprivation, i.e. Nogo 20 would lead to axonal fragmentation. Recently, two groups have started to identify the molecular mechanisms underlying axonal fragmentation. Mok and colleagues have shown that deprivation of NGF in the axonal compartment activates c-jun dependent gene transcription in the cell bodies (Mok et al., 2009). Nikolaev and colleagues identified death receptor 6 (DR6) signaling involving

caspase 6 as the crucial initiation step for axonal fragmentation. Blocking of DR6 delayed fragmentation of sensory axons *in vitro* and the pruning of retinocollicular axons *in vivo* (Nikolaev et al., 2009). Several experimental analyses could be performed to test the role of Nogo-A in axon pruning. As a first step, axonal fragmentation should be analyzed upon Nogo 20 addition in the presence of NGF. If Nogo 20 addition causes axonal fragmentation, the involvement of JNK and DR6 receptor should be carefully explored.

3.3 Final Conclusion

The significance of endocytosis for the signaling of the potent neurite growth inhibitory Nogo-A specific region Nogo 20 that has been demonstrated in this study is just the tip of the iceberg, and unique insights into the Nogo 20 molecular mechanism can probably be obtained from the studies aiming at characterization of the not yet identified high affinity Nogo-A receptor. The characterization of both, Nogo 20 signalosome and the transcriptional changes induced by Nogo 20 endosomes will help to learn more about the antagonistic regulation of neurite growth by neurotrophins and Nogo-A. With the characterization of Nogo 20 signaling endosomes we have just begun to disclose other systems that induce long-range retrograde signaling similar to neurotrophins. Examination of other proteins such as ephrins will reveal whether Pincher-mediated macroendocytosis is a universal mechanism for mediating retrograde endosomal signaling in neurons.

Abbreviations

AA	amino acids
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
AMP	adenosine monophosphate
AP	adaptor protein
BACE-1	β -amyloid converting enzyme 1
BDNF	brain-derived neurotrophic factor
CD	circular dichroism
CLIC	CLathrin-Independent Carriers
CSF	cerebrospinal fluid
CNS	central nervous system
CREB	cAMP response element binding
DIV	day in vitro
dn	dominant negative
DRG	dorsal root ganglion
EEA-1	early endosomal antigen-1
EGF	epidermal growth factor
EH	Eps15 homology
ER	endoplasmatic reticulon
ERK	extracellular signal-regulated kinase
GEEC	GPI-enriched endosomal compartments
GPI	glycosyl-phosphatidylinositol

Abbreviations

GRAF	GTPase regulator associated with focal adhesion kinase
GTP	guanosine triphosphate
LDL	low density lipoprotein
LIMK	LIM kinase
LINGO	Leucine-rich repeats and Ig domain-containing, neurite outgrowth inhibitor (Nogo) receptor-interacting protein-1
MAG	myelin-associated glycoprotein
MAPK	mitogen-activated phosphokinase
MS	multiple sclerosis
NgR	Nogo66 receptor
NMR	nuclear magnetic resonance
NPC	nuclear pore complex
NT-3	neurotrophin-3
OMgP	Oligodendrocyte myelin glycoprotein
Pincher	Pinocytic Chaperon
PI-PLC	phosphatidylinositol-specific phospholipase C
PirB	paired immunoglobulin-like receptor B
PNS	peripheral nervous system
PTRF	polymerase 1 transcript release factor
RBD	Rho binding domain
RTK	receptor tyrosine kinase
RTN	reticulon
SDPR	serum deprivation protein response

Abbreviations

SRF	serum response factor
Trk	tropomyosin-related kinase
wt	wild-type

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ABSTRACTS

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